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54 **Pharmaceutical composition containing a human granulocyte colony stimulating factor for the treatment of leukopenia.**

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CHEMICAL ABSTRACTS, vol. 82, no. 13, 31st March 1975, page 339, column 2, abstract no. 83974n, Columbus, Ohio, US; R.K. SHAD-DUCK "CSF [colony-stimulating factor] response to endotoxin in normal and lenkopenic recipients"

73 Proprietor: Chugai Selyaku Kabushiki Kaisha  
5-1, 5-chome, Ukima Kita-ku  
Tokyo(JP)

72 Inventor: Tamura, Masahiko  
Sahoda roiyaruhaitsu 302 Kuji 14 Takatsu-ku  
Kawasaki-shi Kanagawa-ken(JP)  
Inventor: Nomura, Hitoshi  
3-11-16-218, Igusa Suginami-ku  
Tokyo(JP)  
Inventor: Hattori, Kunihiro  
Chugai inokashiraryo 2-20-9, Inokashira  
Mitaka-shi Tokyo(JP)  
Inventor: Ono, Masayoshi  
1369-7, Yamaguchi  
Tokorozawa-shi Saitama-ken(JP)

74 Representative: Vossius & Partner  
Siebertstrasse 4 P.O. Box 86 07 67  
W-8000 München 86(DE)

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CHEMICAL ABSTRACTS, vol. 90, no. 7, 12th  
February 1979, page 439, column 2, abstract  
no. 52813v, Columbus, Ohio, US; B.L. ZIDAR  
et al.: "Serum colony-stimulating and  
colony-inhibitory activity in response to  
neutropenia"

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## Description

The present invention relates to a leukopenia treating agent which contains a human granulocyte colony stimulating factor (hereinafter abbreviated to human G-CSF) as an effective ingredient.

5 When bone marrow cells as target cells and kidney cells or fetal cells were cultured by the double-layer soft agar cultivation method, with the bone marrow cells being in the upper layer and the kidney or fetal cells in the lower layer, part of the cells in the upper layer grew and differentiated to form colonies of neutrophilic granulocytes (hereunder simply referred to as granulocytes) or monocytic macrophages. This observation has led to the assumption of the presence *in vivo* of factors which promote the formation of  
10 colonies [Pluznik and Sach, *J. Cell. Comp. Physiol.*, 66, 319 (1965); and Bradley and Metcalf, *Aust. J. Exp. Biol. Med. Sci.*, 44, 287 (1966)].

These factors which are collectively referred to as CSF are known to be produced by cells, such as T-cells, monocytic macrophages, fibroblasts and endothelial cells, which normally are distributed extensively *in vivo*. Among subclasses of CSF are included: granulocyte-monocytic macrophage CSF (abbreviated as  
15 GM-CSF) which act on the stem cells of granulocytes or monocyte macrophages in such a manner that they stimulate the growth of such stem cells and induce their differentiation to form colonies of granulocytes or monocytic macrophages; monocytic macrophage CSF (abbreviated as M-CSF) which is principally capable of forming colonies of macrocytic macrophages; multipotent CSF (abbreviated as multi-CSF) which acts on less differentiated multipotent stem cells; and granulocyte CSF (abbreviated as G-CSF) of the type  
20 contemplated by the present invention which is principally capable of forming granulocytic colonies. It has recently been held that the stages of differentiation of target cells differ from one subclass of CSF to another [Asano, *Taisha - Metabolism and Disease*, 22, 249 (1985); and Yunis et al., "Growth and Maturation Factors", edited by Guroff, John Wiley & Sons, NY, vol. 1, 209 (1983)].

Many reports have been published on human CSF, in particular, CSF derived from normal human  
25 tissues and CSF derived from human tumor cells [see, for example, Stanley et al., *Fed. Proc.*, 35, 2272 (1975); Burgess et al., *Blood*, 49, 573 (1977); Shah et al., *Blood* 50, 811 (1977); Fojo et al., *Biochem.*, 17, 3109 (1978); Okabe et al., *Cancer Res.*, 38, 3910 (1978); Asano et al., *Blood*, 49, 845 (1977); Golde et al., *Blood* 57, 1321 (1981); Wu et al., *J. Biol. Chem.*, 254, 6226 (1979); and Dipersio et al., *Blood*, 56, 717 (1980)].

30 However, these species of human CSF are not in a completely pure form and much is left unknown about the utility or effectiveness of human CSF as a pharmaceutical agent.

The number of peripheral leukocytes present in a normal adult ranges from 4000 to 9000 per mm<sup>3</sup> [see Takigawa et al., *Rinsho Kensa (Clinical Examination)*, 12, 12, 906 - 910, 1968], but for various reasons, their number can drop below 4000 and this abnormal state is generally referred to as leukopenia.

35 Leukopenia sometimes occurs as a result of uniform decrease in the number of various types of leukocytes but, more often than not, the disease is due to a decrease in the number of a particular type of leukocyte and may be classified as neutropenia, eosinopenia and lymphopenia according to the type of the leukocyte that has decreased in number. However, on a clinical basis, most cases of leukopenia are due to a decrease in the number of neutrophiles. The mechanism behind the decrease in the number of  
40 neutrophiles is two-fold: 1) lowered production of neutrophiles in the bone marrow; and 2) enhanced consumption and destruction of neutrophiles in the peripheral blood. While many causative diseases or lesions [such as essential ones that are caused by unknown factors and those which accompany exposure to radiation or a variety of blood diseases; for details, see Shindan to Chiryō (Diagnosis and Treatment), 70,  
7, 24 - 31, 1982] are known to be responsible for these phenomena, most cases of neutropenia have  
45 occurred on account of the administration of various drugs and, in particular, the drug-induced decrease in the number of neutrophiles which can potentially lead to so-called agranulocytosis accompanied by a decrease in the absolute number of neutrophiles to 150/mm<sup>3</sup> or below [see, for example M. Komine, *Ketsuekibyogaku (Hematology)*, p. 227 and p. 849, 1981] has clinically become a matter of great concern.

Many drugs can cause a decrease in the number of neutrophiles and are generally classified as two  
50 types: those which cause pancytopenia such as alkylating agents (e.g. cyclophosphamide), metabolic antagonists (cytosine arabinoside) and cancer-controlling antibiotics (e.g. daunorubicin); and those which reduce the number of neutrophiles by so-called drug hypersensitivity, such as sulfa agents, antipyretic analgesic sedatives (e.g. aminopyrine), anticonvulsants, antibiotics (e.g. chloramphenicol) and antithyroid agents [for details, see Shindan to Chiryō (Diagnosis and Treatment), 70, 7, pp 24-31, 1981].

55 The number of neutrophiles can be increased by such drugs as cepharanthin, adenine agents, L-cystine, parotin and the decomposition products of ribonucleic acids. However, these drugs are not completely satisfactory for clinical purposes because of their low effectiveness or because of the high incidence of side effects.

In addition, it is required that the neutrophils which are restored or increasing in number should assume a sufficiently mature form to possess their inherent capabilities; otherwise, they would not be able to achieve the defense mechanism such as phagocytic action that is necessary for maintaining the normal state of a living organism.

Therefore, it has long been desired to develop a drug that is capable of increasing the number of neutrophils having a fully mature form and which yet causes a reduced incidence of side effects.

To meet this demand, the assignee of the present invention established from the tumor cells of patients with oral cavity cancer a cell strain, CHU-1 (C.N.C.M. Accession Number I-315), that had an extremely high capability of human G-CSF production and which exhibited a high capacity for growth. The assignee cultured this cell and successfully isolated from the supernatant of the culture a pure human G-CSF that displayed an activity for accelerating the formation of colonies of human neutrophils (Japanese Patent Application No. 153273/1984). Subsequently, the assignee established a cell strain, CHU-2 (C.N.C.M. Accession Number I-483) from human oral cavity cancer, cultured this cell and also succeeded in isolating, from the supernatant of the culture, a G-CSF which was completely identical to the one derived from CHU-1.

The assignee continued their studies with a view to developing a method for G-CSF production by recombinant DNA technology that is capable of attaining a higher concentration of G-CSF than the previous methods of cell cultivation without requiring any complicated purifying procedures and, as a result, they succeeded in large-scale preparation of a homogenous human G-CSF by recombinant DNA technology (Japanese Patent Application Nos. 269455/1985, 269456/1985, 270838/1985 and 270839/1985).

On the basis of these accomplishments, the inventors of the present invention administered the above-described human G-CSF to normal animals and models that were suffering from a decrease in the number of neutrophils. This factor caused a significant increase in the number of neutrophils in the normal animals whereas it displayed an ability to inhibit to a significant extent the decrease in the number of neutrophils in the neutropenic animal models. In addition, the increased neutrophils assumed a fully mature form. Therefore, the present inventors found that the human G-CSF could be used as an effective leukopenia treating agent. The present invention has been accomplished on the basis of this finding.

Fig. 1 shows the amino acid and the nucleotide sequences of three different probes, IWQ, A and LC;

Fig. 2 shows the nucleotide sequence of a cDNA insert in pBRG4;

Fig. 3 shows the nucleotide sequence of a cDNA insert in pBRV2;

Fig. 4 is a presentation of the process for constructing an expression recombinant vector pTN-G4;

Fig. 5 shows a process for constructing an expression recombinant vector pHGG4-dhfr;

Fig. 6 is a presentation of the process for constructing an expression recombinant vector pTN-V2;

Fig. 7 shows a process for constructing an expression recombinant vector pHGV2-dhfr.

The present invention provides a novel leukopenia treating agent containing human G-CSF as an active ingredient.

Any human G-CSF that has been purified to an extent that makes it suitable for use in a leukopenia treating agent may be employed as the active ingredient of the leukopenia treating agent of the present invention. Preferable human G-CSFs are ones obtained by isolation from the supernatant of the culture of a human G-CSF producing cell, and a polypeptide or glycoprotein having the human G-CSF activity that is obtained by transforming a host with a recombinant vector having incorporated therein a gene coding for a polypeptide having the human G-CSF activity.

Two particularly preferably examples of human G-CSF are shown below:

(1) human G-CSF having the following physicochemical properties:

i) molecular weight: about  $19,000 \pm 1,000$  as measured by electrophoresis through a sodium dodecylsulfate - polyacrylamide gel;

ii) isoelectric point: having at least one of the three isoelectric points,  $pI = 5.5 \pm 0.1$ ,  $pI = 5.8 \pm 0.1$ , and  $pI = 6.1 \pm 0.1$ ;

iii) ultraviolet absorption: having a maximum absorption at 280 nm and a minimum absorption at 250 nm;

iv) amino acid sequence of the 21 residues from N terminus:  $H_2N$ -Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-

(2) human G-CSF containing either a polypeptide having the human granulocyte stimulating factor activity which is represented by all or part of the amino acid sequence shown below, or a glycoprotein having both said polypeptide and a sugar chain portion:

(Met)<sub>n</sub> Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro  
 Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val  
 5 Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
 Glu Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys  
 Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly  
 10 His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser  
 Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly  
 Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu  
 Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile  
 15 Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu  
 Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile  
 Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro  
 20 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala  
 Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu  
 25 Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala  
 Gln Pro

(provided that m is 0 or 1; and n is 0 or 1).

The human G-CSFs (1) and (2) may be produced by the methods described in the Referential  
 30 Examples shown later in this specification. Stated more specifically, human G-CSF (1) can be prepared by  
 the methods described in Referential Examples 1 and 2, human G-CSF (2) by the methods described in  
 Referential Examples 4 to 11.

For details of the conditions employed for performing these methods, see the specification of Japanese  
 Patent Application Nos. 153273/1984, 269455/1985, 269456/1985, 270838/1985 and 270839/1985, all having  
 35 been filed by the assignee of the present invention.

Another method that can be employed consists of performing fusion of a G-CSF producing cell with a  
 self-proliferating malignant tumor cell and cultivating the resulting hybridoma in the presence or absence of  
 mitogen.

All of the human G-CSFs that are prepared by the methods described above are included within the  
 40 scope of the present invention.

The human G-CSF containing solution obtained may be stored in a frozen state after being further  
 purified and concentrated, as required, by any known technique. Alternatively, the solution may be stored  
 after being dehydrated by such means as freeze-drying or vacuum drying. If desired, the human G-CSF  
 may be dissolved in an appropriate buffer, followed by aseptic filtration through a Millipore filter or any other  
 45 suitable means so as to formulate an injection.

The leukopenia treating agent of the present invention may contain a pharmaceutical carrier or excipient  
 necessary to assist in its formulation in a dosage form suitable for administration to humans or animals. If  
 desired, a stabilizer and an anti-adsorption agent may also be incorporated in the agent.

The level of dosage and the frequency of administration of the human G-CSF in the leukopenia treating  
 50 agent of the present invention may be determined in consideration of the severity of the disease to be  
 treated; typically, a dosage containing 0.01 - 500 µg, preferably 0.5 - 200 µg, of human G-CSF may be  
 administered to an adult in one to seven portions over a week. However, it should be noted that the present  
 invention is by no means limited with respect to the content of human G-CSF.

#### 55 Examples

The following referential examples and working examples are provided for the purpose of further  
 illustrating the present invention but should in no way be taken as limiting.

Referential Example 1: Assaying CSF Activity

The following methods were used to determine the CSF activity (hereunder abbreviated as CSA) in the present invention.

5

CSA assay

(a) With human bone marrow cells:

10 Single-layer soft agar cultivation was conducted in accordance with the method of Bradley, T.R. and Metcalf, D. (Aust. J. Exp. Biol. Med. Sci., 44, 287-300, 1966). More specifically, 0.2 ml of a bovine fetal serum, 0.1 ml of the sample, 0.1 ml of a human bone marrow nonadherent cell suspension ( $1 - 2 \times 10^5$  nuclear cells), 0.2 ml of a modified McCoy's 5A culture solution, and 0.4 ml of a modified McCoy's 5A culture solution containing 0.75% of agar were mixed, poured into a plastic dish for tissue culture (35 mm<sup>ø</sup>),  
 15 coagulated, and cultured at 37 °C in 5% CO<sub>2</sub>/95% air and at 100% humidity. Ten days later, the number of colonies formed was counted (one colony consisting of at least 50 cells) and CSA was determined with one unit being the activity required for forming one colony.

(b) With mouse bone marrow cells:

20

A horse serum (0.4 ml), 0.1 ml of the sample, 0.1 ml of a C3H/He (female) mouse bone marrow cell suspension ( $0.5 - 1 \times 10^5$  nuclear cells), and 0.4 ml of a modified McCoy's 5A culture solution containing 0.75% of agar were mixed, poured into a plastic dish for tissue culture (35 mm<sup>ø</sup>), coagulated, and cultured for 5 days at 37 °C in 5% CO<sub>2</sub>/95% air and at 100% humidity. The number of colonies formed was counted  
 25 (one colony consisting of at least 50 cells) and CSA was determined with one unit being the activity for forming one colony.

The modified McCoy's 5A culture solution used in each of the methods (a) and (b) and the human bone marrow nonadherent cell suspension used in (a) were prepared by the following procedures.

30 Modified McCoy's 5A culture solution (double concentration)

Twelve grams of McCoy's 5A culture solution (Gibco), 2.55 g of MEM amino acid-vitamin medium (Nissui Seiyaku Co., Ltd.), 2.18 g of sodium bicarbonate and 50,000 units of potassium penicillin G were dissolved twice in 500 ml of distilled water and the solution was aseptically filtered through a Millipore filter  
 35 (0.22 μm).

Human bone marrow nonadherent cell suspension

A bone marrow fluid obtained from a healthy person by sternal puncture was diluted 5-fold with an RPMI 1640 culture solution, plated over a Ficoll-Paque solution (Pharmacia Fine Chemicals) and centrifuged at 400 x g for 30 minutes at 25 °C. The interfacial cell layer (specific gravity <1.077) was recovered. The cells were washed, adjusted to a concentration of  $5 \times 10^6$  cells/ml with an RPMI 1640 culture solution containing 20% of bovine fetal serum, poured into a 25-cm<sup>2</sup> plastic flask for tissue culture, and incubated for 30 minutes in a CO<sub>2</sub> incubator. Nonadherent cells were recovered in the supernatant, poured into a plastic  
 45 flask (25 cm<sup>2</sup>) and incubated for 2 hours and a half. Nonadherent cells in the supernatant were collected and used in an assay.

Referential Example 2: Isolation of Human G-CSF

## 50 (1) Establishment of CHU-2

A tumor of a patient with oral cavity cancer wherein pronounced increase was observed in the number of neutrophils was transplanted into nu/nu mice. About 10 days after the transplantation, the increase in the weight of the tumor and in the number of neutrophils was significant. Twelve days after the transplantation, the tumor was extracted aseptically, dissected into cubes of 1 - 2 mm<sup>3</sup> and cultured in the following  
 55 manner.

Ten to fifteen cubes of the tumor were put into a 50-ml plastic centrifugal tube. After addition of 5 ml of a trypsin solution (containing 0.25% of trypsin and 0.02% of EDTA), the tube was shaken for 10 minutes in

a warm bath at 37 °C and the supernatant was discarded. Another 5 ml of the same trypsin solution was added and trypsin digestion was conducted under agitation for 15 minutes at 37 °C. The supernatant cell suspension was recovered and stored in ice after the trypsin had been inactivated by addition of 1 ml of a bovine fetal serum.

5 After repeating these procedures once again, the cell suspension was recovered, combined with the previously obtained suspension, and centrifuged at 400 x g for 10 minutes to obtain a cell pellet. The pellet was washed twice with F-10 containing 10% of a bovine fetal serum and was thereafter loaded in a plastic culture flask (25 cm<sup>2</sup>) to give a cell concentration of 5 x 10<sup>6</sup> cells/flask. After incubation overnight in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> and 100% humidity) with an F-10 culture solution containing 10% of a bovine fetal serum, the supernatant was removed together with the nonadherent cells, and culture was continued with a fresh supply of culture solution. Six days after the start of culture, the flask became full of the cells and the culture solution was replaced by a fresh one. On the next day, the culture solution was discarded and the flask was charged with 2 ml of an anti-mouse erythrocyte antibody (Cappel) diluted 5-fold with RPMI 1640 and 2 ml of a guinea pig complement (Kyokuto Seiyaku Co., Ltd.) diluted 2.5-fold with RPMI 1640. After incubation for 20 minutes at 37 °C, the culture was washed twice with F-10 containing 10% of a bovine fetal serum and the nu/nu mouse derived fibroblasts were removed. Subsequently, an F-10 culture solution containing 10% of a bovine fetal serum was added and cultivation was conducted for 2 more days. Thereafter, some of the cells were recovered and subjected to cloning by the limiting dilution method.

20 The resulting 11 clones were checked for their CSF activity and one clone (CHU-2) exhibited activity about 10 times as high as that of the other clones.

## (2) Isolation of Human G-CSF

25 The cells established in (1) were grown in a completely dense population (grown to confluency) in two culture flasks (150 cm<sup>2</sup>). The cells were recovered, suspended in 500 ml of an F-10 culture solution containing 10% of a bovine fetal serum, transferred into a glass roller bottle of 1580 cm<sup>2</sup> (Belco), and whirl-cultured at 0.5 rpm. When the cells were found to have grown in a completely dense population (grown to confluency) on the inner wall of the roller bottle, the culture solution was replaced by a serum-free RPMI 1640. After 4-day culture, the supernatant of the culture was recovered and cultivation was continued with F-10 containing 10% of a bovine fetal serum being added. After 3-day culture, the culture solution was again replaced by a serum-free RPMI 1640 and the supernatant of the culture was recovered 4 days later. By repeating these procedures, 500 ml of the serum-free supernatant of culture per bottle was obtained each week. In addition, this method enabled the supernatant of culture to be recovered, with the cells maintained over a significantly prolonged period.

35 A batch consisting of 5,000 ml of the supernatant of the culture obtained was mixed with 0.01% of Tween 20 and concentrated about 1000 times by ultrafiltration with Hollow Fiber DC-4 and Amicon PM-10 (Amicon). The concentrate was purified by the following steps.

40 (i) A portion (5 ml) of the concentrated supernatant of culture was subjected to gel filtration on an Ultrogel ACA54 column (4.6 cm<sup>φ</sup> x 90 cm<sup>h</sup>; LKB) at a flow rate of ca. 50 ml/hr with 0.01 M Tris-HCl buffer (pH 7.4) containing .015 M NaCl and 0.01% Tween 20 (Nakai Kagaku Co., Ltd.) The column had been calibrated with bovine serum albumin (Mw; 67,000), ovoalbumin (Mw; 45,000) and cytochrome C (Mw; 12,400). After completion of the gel filtration, 0.1 ml of each of the fractions was diluted 10-fold and screened for the active fractions by the above-described method of CSA assay (b). The fractions for V<sub>e</sub> = 400 - 700 ml were found to exhibit macrophage-dominant CSA while the fractions for V<sub>e</sub> = 800 - 1200 ml showed granulocyte-dominant CSA. Therefore, the latter fractions were collected and concentrated to a volume of ca. 5 ml on an ultrafiltration apparatus with PM-10 (Amicon).

45 (ii) To the cocentrated fractions was added an aqueous solution of 0.1% trifluoroacetic acid containing 30% of n-propanol (for determination of amino acid sequence; available from Tokyo Kasei K.K.) After the mixture had been left to stand in ice for about 15 minutes, the precipitate was removed by centrifugation for 10 minutes at 27,000 x g. The supernatant was adsorbed on a μ-Bondapak C18 column (8 mm x 30 cm for semipreparatory use; Waters) equilibrated with the aqueous solution containing n-propanol and trifluoroacetic acid; the column was continuously eluted with an aqueous solution of 0.1% trifluoroacetic acid which contained n-propanol having a linear concentration gradient of 30 - 60%. A high performance liquid chromatographic apparatus, Hitachi Model 685-50 (Hitachi, Ltd.), and a detector, Hitachi Model 638-41 (Hitachi, Ltd.) were employed to determine the absorptions at 220 nm and 280 nm simultaneously. After elution, 10 μl of each of the fractions was diluted 100-fold and screened for the active fractions by the above-described method of CSA assay (b). The peaks eluted with 40% n-propanol were found to have CSA activity, so they were collected, re-chromatographed under the same conditions, and

assayed for CSA by the same method. Again, CSA activity was observed in the peaks at 40% n-propanol. Therefore, these peaks were collected (4 fractions = 4 ml) and freeze-dried.

(iii) The freeze-dried powder was dissolved in 200  $\mu$ l of an aqueous solution of 0.1% trifluoroacetic acid containing 40% of n-propanol, and the solution was subjected to high performance liquid chromatography on TSK-G 3000SW column (Toyo Soda Manufacturing Co., Ltd.; 7.5 mm x 60 cm). Elution was conducted with the same aqueous solution at a flow rate of 0.4 ml/min and the fractions were taken in 0.4-ml portions with a fraction collector, FRAC-100 (Pharmacia Fine Chemicals). Each of the fractions taken was checked for its CSA by the same method as described above and activity was observed in the fractions for retention times of 37 - 38 minutes (corresponding to MW of ca.  $2 \times 10^4$ ). The active fractions were recovered and purified on an analytical  $\mu$ -Bondapak C18 column (4.6 mm x 30 cm). The main peaks were recovered and freeze-dried. The sample obtained was assayed by the method of CSA assay (a); it was found to have human G-CSF activity.

### Referential Example 3: Physicochemical Properties

The physicochemical properties of human G-CSF which was prepared in Referential Example 2 were determined by the following analyses and test.

#### (i) Molecular weight

The molecular weight of the CSF was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic equipment was PROTEAN<sup>TM</sup> (16 cm, product of Bio-Rad Corporation), using a gel made up of a polyacrylamide slab gel (T = 15%, C = 2.6%) measuring 140 mm x 160 mm x 1.5 mm, and a concentrating gel (T = 3%, C = 20%). A denatured CSF sample was prepared by the following procedure: CSF was boiled for 3 minutes in a solution containing 2% of sodium dodecylsulfate in 0.46 M 2-mercaptoethanol. After performing electrophoresis with 4  $\mu$ g of the sample with a constant current of 30 mA for 4 hours, the gel was removed and stained with 0.25% Coomassie Brilliant Blue R 250 (product of Sigma Chemical Co.) for band detection. The following substances were used as molecular weight markers after similar treatments: phosphorylase B (mol. wt. 92,500), bovine serum albumin (BSA, mol. wt. 67,000), ovalbumin (OVA, mol. wt. 45,000), carbonic anhydrase (mol. wt. 31,000), soybean trypsin inhibitor (mol. wt. 21,500) and lysozyme (mol. wt. 14,400). A single band corresponding to a molecular weight of approximately 19,000 was detected.

In view of the result, the human G-CSF of the present invention is deemed to have a molecular weight of  $19,000 \pm 1,000$ .

#### (ii) Isoelectric point

The isoelectric point of the CSF of the present invention was determined by a flat bed, isoelectric electrophoretic apparatus, FBE-3000 (product of Pharmacia Fine Chemicals). After 2-hr electrophoresis with a constant power of 30 watts ( $V_{max} = 2,000$  volts) on a polyacrylamide gel (T = 5%, C = 3%, 115 mm x 230 mm) containing Pharmalyte (pH = 4 - 6.5, Pharmacia Fine Chemicals) and 4M urea, the CSF was fixed with 30% methanol/10% trichloroacetic acid/35% sulfosalicylic acid, and stained with Coomassie Brilliant Blue R-250. A Low pI kit (pH: 2.5 - 6.5, product of Pharmacia Fine Chemicals) was used as an isoelectric point marker.

Analysis of band separation in the pH range of 4 to 6.5 gave three distinct bands corresponding to pI = 5.52, 5.80 and 6.13, among which the two bands for pI = 5.52 and 5.80 were predominant components.

Another five measurements of the same sample of CSF gave the following results: pI =  $5.5 \pm 0.1$ ,  $5.8 \pm 0.1$  and  $6.1 \pm 0.1$ .

#### (iii) UV absorption

The sample was checked for its UV absorption with a spectrophotometer, with 0.1% trifluoroacetic acid containing 40% of n-propanol being taken as a reference. A maximum peak occurred at 280 nm and a minimum peak at 250 nm. (iv) Determination of amino acid sequence

#### ① Determination of N-terminal amino acid sequence

The sample was subjected to Edman degradation with a gas-phase sequencer (Applied Biosystems)



and the resulting PTH amino acid was analyzed by routine procedures with a high performance liquid chromatographic apparatus (Beckman Instruments) and Ultrasphere-ODS column (Beckman Instruments). The column (5  $\mu\text{m}$ ; 4.6 mm $\phi$  x 250 mm $^{\text{L}}$ ) was equilibrated with a starting buffer [aq. sol. containing 15 mM sodium acetate buffer (pH 4.5) and 40% acetonitrile] and injected with the sample (as dissolved in 20  $\mu\text{l}$  of the starting buffer). Separation was effected by isocratic elution with the starting buffer. The flow rate was 1.4 ml/min and the column temperature was held at 40 $^{\circ}\text{C}$ . Detection of the PTH amino acid was achieved utilizing the absorptions in the UV range at 269 nm and 320 nm. Standard samples of PTH amino acid (Sigma) in 2-nmol portions were separated on the same line to determine their retention times, which were compared with those of the sample to be tested. As a result, the sample was found to have the following amino acid sequence of the 40 residues from N-terminus:

$\text{H}_2\text{N}$  - Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser -  
 (10)  
 Leu - Pro - Gln - Ser - Phe - Leu - Leu - Lys - Cys -  
 (20)  
 Leu - Glu - Gln - Val - Arg - Lys - Ile - Gln - Gly -  
 (30)  
 Asp - Gly - Ala - Ala - Leu - Gln - Glu - Lys - Leu -  
 (40)  
 Cys - Ala - Thr - Tyr - Lys -

### ② Degradation with bromocyan

The sample was dissolved in 70% formic acid. To the solution, 200 equivalent amounts of bromocyan that had been purified by sublimation was added. The mixture was left overnight at 37 $^{\circ}\text{C}$  for reaction. The reaction product was freeze-dried and fractionated by HPLC on a TSK G3000SW column (Toyo Soda Manufacturing Co., Ltd.) to obtain four peaks. The peaks were named CN-1, CN-2, CN-3 and CN-4 in the decreasing order of the molecular weight. The first two peaks (CN-1 and CN-2) had better yields and their amino acid sequences were analyzed with an automatic gas-phase sequencer (Applied Biosystems) under the same conditions as used in ①.

As a result, CN-1 was found to be a peptide from the N-terminus of G-CSF protein, and CN-2 had the following amino acid sequence:

Pro - Ala - Phe - Ala - Ser - Ala - Phe -  
 Gln - Arg - Arg - Ala - Gly - Gly - Val -  
 Leu - Val - Ala - Ser - His - Leu - Gln -

### ③ Digestion with trypsin

The sample was dissolved in 0.1 M Tris-HCl buffer (pH 7.4) containing 8 M urea and the solution was mixed with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1% 2-mercaptoethanol to provide a final urea concentration of 2 M. A TPCK-treated trypsin (Sigma) was added such that the sample-to-enzyme ratio was 50:1. The mixture was held for 4 hours at 25 $^{\circ}\text{C}$  and, after addition of an equal amount of TPCK-treated trypsin, the mixture was held for an additional 16 hours at 25 $^{\circ}\text{C}$ . Thereafter, the reaction product was subjected to high-speed reverse-phased column chromatography on C8 column (Yamamura Kagaku K.K.), with elution conducted with 0.1% TFA containing n-propanol having a linear density gradient of 5 - 60%. While several peaks were obtained by measuring the absorption at 280 nm, the main peak was analyzed for its amino acid sequence with an automatic gas-phase sequencer (Applied Biosystems) under the same conditions as used in ①. As a result, the main peak was found to be a peptide having the following sequence which contained part of the CN-2 fragment shown in ②:

Gln - Leu - Asp - Val - Ala - Asp - Phe - Ala - Thr -  
 Thr - Ile - Trp - Gln - Gln - Met - Glu - Glu - Leu -  
 Gly - Met - Ala - Pro - Ala - Leu - Gln - Pro - Thr -  
 Gln - Gly - Ala - Met - Pro - Ala - Phe - Ala - Ser -

10 (v) Amino acids in the protein portion

The sample was hydrolyzed by a conventional method and analyzed for the amino acid composition of the protein portion with an automatic amino acid analyzer, Model 835 of Hitachi, Ltd. The results are shown in Table 1. The hydrolysis was conducted under the following conditions:

- 15 ① 6 N HCl, 110° C, 24 hours, in vacuum  
 ② 4 N methanesulfonic acid + 0.2% 3-(2-aminoethyl)indole, 110° C, 24 hours, 48 hours, 72 hours, in vacuum

20 The sample was dissolved in a solution (1.5 ml) containing 40% n-propanol and 0.1% trifluoroacetic acid. Aliquots each weighing 0.1 ml were dried with a dry nitrogen gas and, after addition of the reagents listed in ① or ②, the containers were sealed in vacuum, followed by hydrolysis of the contents.

Each of the values shown in Table 1 was the average of four measurements, 24 hour value for ① and 24, 48 and 72 hour values for ②, except that the contents of Thr, Ser, 1/2 Cys, Met, Val, Ile and Trp were calculated by the following methods [see "Tampaku Kagaku (Protein Chemistry) II", A Course in Biochemical Experiments, Tokyo Kagaku Dohjin]:

- 25 - - For Thr, Ser, 1/2 Cys and Met, the time-dependent profile of the 24, 48 and 72 hour values for ② was extrapolated by zero hours.  
 - - For Val and Ile, the 72 hour value for ② was used.  
 - - For Trp, the average of 24, 48 and 72 hour values for ② was used.

30 The values shown in the column of "Predicted Number of Residual Amino Acid Groups" are based on the assumption of the existence of 33 Leu's. Generally, the amino acids that require correction of the type described above are decomposed either somewhat or substantially during hydrolysis or they are resistant to hydrolysis. In particular, Pro Produces a low color yield. Primarily for these reasons, the actually measured contents (nmol) of amino acids of interest and hence, the calculated number of each of the residues, has a tendency to be lower than theoretical values (see Seikagaku Jikken Koza, ibid).

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Table 1

| Amino acids  | Measured values (nmol) | Predicted number of amino acid residues (rounded to the parenthesized integrals) |
|--|------------------------|--|
| Asp (Asp + Asn)  | 3.54                   | 4.3 (4)  |
| Thr  | 4.58                   | 5.5 (6)  |
| Ser  | 10.64                  | 12.9 (13)  |
| Glu (Glu + Gln)  | 22.31                  | 27.0 (27)  |
| Pro  | 8.30                   | 10.1 (10)  |
| Gly  | 10.60                  | 12.8 (13)  |
| Ala  | 14.85                  | 18.0 (18)  |
| 1/2 Cys  | 2.59                   | 3.1 (3)  |
| Val  | 6.16                   | 7.5 (7)  |
| Met  | 2.26                   | 2.7 (3)  |
| Ile  | 3.29                   | 4.0 (4)  |
| Leu  | 27.24                  | 33.0 (33)  |
| Tyr  | 2.60                   | 3.1 (3)  |
| Phe  | 5.08                   | 6.1 (6)  |
| Lys  | 3.68                   | 4.5 (4)  |
| His  | 3.93                   | 4.8 (5)  |
| Trp  | 1.61                   | 1.9 (2)  |
| Arg  | 4.29                   | 5.2 (5)  |
| Total  |                        | (166)  |
| Calculated molecular weight (no sugar counted in 166 residues) |                        | 17961  |

#### 30 Referential Example 4: Probe Preparation

Based on the amino acid sequences determined in Referential Example 3 (iv), three nucleotide probes, (A), (LC) and (IWQ) were synthesized. Probe (A) was of the mixed type composed of 14 successive nucleotides. Probe (IWQ) was composed of 30 successive nucleotides with deoxyinosine and was a probe of the type used in the cloning of the human cholecystokinin gene [Takahashi et al., Proc. Natl. Acad. Sci., USA, 82, 1931 (1985)]. Probe (LC) was a 24-nucleotide probe that was synthesized on the basis of the nucleotide sequence shown in Fig. 2; it had a nucleotide sequence which corresponds to the nucleotide sequence at position 32 to 39 from the N terminus of the amino acid sequence shown in Referential Example 3 (iv)-①.

40 Chemical synthesis of nucleotides can be achieved by applying the improved phosphotriester method to the solid phase method and has been reviewed by Narang [Tetrahedron, 39, 3-22 (1983)].

Probes based on amino acid sequences at positions other than those in the above-mentioned probes may also be used.

#### 45 Referential Example 5: Construction of cDNA Library

CHU-2 cells were homogenized after addition of a guanidine thiocyanate solution and the total RNA was obtained by CsCl density gradient centrifugation.

50 Poly (A<sup>+</sup>) RNA was isolated from the total RNA by column chromatography on oligo(dT)-cellulose. Thereafter, a single-stranded cDNA was synthesized with a reverse transcriptase, and RNase H and E. coli DNA polymerase I were added to obtain a double-stranded cDNA. A dC chain was attached to the obtained double-stranded cDNA, which was joined to a vector, pBR322, to which a dG chain had been attached at the Pst I cleavage site. The resulting recombinant DNA was used to transform a strain of E. coli, X1776, and a pBR322-line cDNA library was constructed.

55 In a similar manner, the double-stranded cDNA was joined to the λgt10 vector with the EcoRI linker and λ-phage line cDNA library was constructed.

#### Referential Example 6: Screening

Two line of cDNA were obtained by the following procedures.

Recombinants derived from the pBR322-line cDNA library were fixed on Whatmann 541 filter paper and a single clone could be selected by colony hybridization with <sup>32</sup>p-labelled probe (IWQ). Further study with the Southern blotting method [Southern, J. Mol. Biol., 98, 503 (1975)] showed that this clone also hybridized with probe (A). The nucleotide sequence of this clone was determined by the dideoxy method [Sanger, Science, 214, 1205 (1981)].

The obtained cDNA insert was found to consist of 308 base pairs including probes (IWQ) and (A), and have an open reading frame coding for 83 amino acids containing the amino acid sequence shown in Referential Example 3 (iv)- ③. The pBR322-derived plasmid containing these 308 base pairs is hereunder referred to as pHCS-1.

A DNA fragment containing the 308 base pairs obtained from pHCS-1 was radiolabelled by the nick translation method (see Molecular Cloning, *ibid.*) and, with this fragment used as a probe, the λgt10-derived cDNA library was screened by plaque hybridization [Benton and Davis, Science, 196, 180 (1977)] to obtain five clones. The nucleotide sequence of a clone which was believed to contain cDNA was determined by the same method as described above (Fig. 2).

As shown in Fig. 2, this cDNA insert had a single large open reading frame.

The amino acid sequence encoded by this cDNA can be deduced as shown in Fig. 2.

The cDNA of this line is hereinafter referred to as cDNA (+VSE).

Comparison with then N-terminal amino acid sequence of G-CSF protein shown in Example 3 (iv)- ① revealed that this cDNA contained a nucleotide sequence which corresponded to both a signal peptide encoded by 90 base pairs starting with the ATG sequence at 32 - 34 nucleotide positions from 5'-terminus and ending with the GCC sequence at 119 - 121 positions, and a mature G-CSF polypeptide encoded by 531 base pairs starting with the ACC sequence at 122 - 124 positions and ending with the CCC sequence at 650 - 652 positions. Therefore, the precursor polypeptide which comprised the signal peptide and the mature G-CSF polypeptide was composed of 207 amino acids and its molecular weight was calculated as 22292.67 daltons. The mature G-CSF polypeptide was composed of 177 amino acids and its molecular weight was calculated as 18986.74 daltons.

It should be noted that the ATG at 32 - 34 positions or at 68 - 70 positions can also be considered to be the protein initiation site. *Escherichia coli* strain X1776 R-1 harboring pBR322 which had this cDNA (+VSE) at the EcoRI cleavage site has been deposited with the Fermentation Research Institute, the Agency of Industrial Science and Technology (FERM BP-954).

This cDNA was joined to pBR327 [Soberon et al., Gene, 9, 287 (1980)] at the EcoRI site and the resulting plasmid is hereunder referred to as pBRG4.

The thus obtained pBRG4 was treated with a restriction enzyme, EcoRI, to obtain a DNA fragment containing cDNA of about 1500 base pairs. This fragment was radiolabelled by the nick translation method (see Molecular Cloning, *ibid.*) and, with this radiolabelled DNA fragment being used as a probe, the λgt10-derived cDNA library was screened once again by plaque hybridization (see Benton and Davis, *ibid.*) In this plaque hybridization, two sheets of λ-phage DNA fixed nitrocellulose filter paper were prepared; one of these sheets was used for the above-mentioned plaque hybridization and another one was subjected to plaque hybridization with the already described probe (LC). The phages which turned positive for both probes were selected. A clone which has a "full-length" cDNA was selected and the nucleotide sequence of the cDNA insert as determined by the dideoxy method is shown in Fig. 3.

This cDNA had a single large open reading frame and the amino acid sequence that would be encoded by this cDNA was deduced as shown in Fig. 3.

The cDNA of this line is hereinafter referred to as cDNA (-VSE).

Comparison with the N-terminal amino acid sequence of G-CSF protein shown in Example 3 (iv)- ① revealed that this cDNA contained a nucleotide sequence which corresponded to both a signal peptide encoded by 90 base pairs starting with the ATG sequence at 31 - 33 nucleotide positions from 5'-terminus and ending with the GCC sequence at 118 - 120 positions, and a mature G-CSF polypeptide encoded by 522 base pairs starting with the ACC sequence at 121 - 123 positions and ending with the CCC sequence at 640 - 642 positions. Therefore, the precursor polypeptide which comprised the signal peptide and the mature G-CSF polypeptide was composed of 204 amino acids and its molecular weight was calculated as 21977.35 daltons. The mature G-CSF polypeptide was composed of 174 amino acids and its molecular weight was calculated as 18671.42 daltons.

It should be noted that ATG at 58 - 60 positions or at 67 - 69 positions can also be considered to be the protein initiation site.

*Escherichia coli* strain X1776 R-2 harboring pBR322 which had this cDNA (-VSE) at the EcoRI cleavage site has been deposited with the Fermentation Research Institute, the Agency of Industrial Science and

Technology (FERM BP-955).

This cDNA was joined to pBR327 at the EcoRI site to form a plasmid which is hereunder referred to as pBRV2.

5 Referential Example 7: Construction of Recombinant Vector for Expression in E. coli

(A) +VSE line recombinant vector

From the pBRG4 plasmid obtained in Referential Example 6, a cDNA fragment of the G-CSF  
10 polypeptide was cut out with a restriction enzyme and a recombinant vector was constructed by one of the following methods:

- (i) using an annealed synthetic linker, the fragment was ligated with a fragment prepared from a tac promoter-containing pKK223-3 (Pharmacia Fine Chemicals);
- 15 (ii) three fragments prepared from P<sub>L</sub> promoter containing pPL-lambda (Pharmacia Fine Chemicals) were ligated with an annealed synthetic linker and, the ligation product and the cDNA fragment were subjected to re-preparation procedures to construct a recombinant vector; or
- (iii) using an annealed synthetic linker, the fragment was ligated with a fragment prepared from a trp promoter-containing pOY1 plasmid.

20 (B) -VSE line recombinant vector

In the same manner as described above, three recombinant vectors were constructed using the plasmid pBRV2 obtained in Referential Example 6.

25 Referential Example 8: Preparation of E. coli Transformants, and Cultivation and Expression Thereof

Using three recombinant vectors of each of the +VSE and -VSE lines, E. coli strain DH1, N4830 or JM105 was transformed by the calcium chloride or rubidium chloride procedure described in Molecular Cloning, *ibid.* Each of the transformants obtained was cultivated in ampicillin-containing Luria medium, with  
30 induction being subsequently conducted as required to achieve expression.

Referential Example 9: Recovery and Purification of G-CSF Polypeptide from E. coli and Amino Acid Analysis Thereof

35 A culture solution of the transformants was centrifuged to obtain a cell pellet. The collected cells were treated with lysozyme and, after lysis by cyclic freezing and thawing, the supernatant was obtained. Alternatively, the cells were treated with guanidium chloride, centrifuged and the supernatant was recovered.

The supernatant was subjected to gel filtration on an Ultrogel ACA54 column (LKB) and the active fractions were concentrated with an ultrafiltration apparatus.

40 Subsequently, an aqueous solution of trifluoroacetic acid containing n-propanol was added to the concentrate and, after being left in ice, the mixture was centrifuged and adsorbed on a reverse-phase C18 column. After elution, the fractions were checked for their activity. The active fractions were collected and subjected to the same procedures of purification as described above. The purified fractions were freeze-dried and the powder was dissolved and subjected to high performance liquid chromatography based on  
45 molecular size. The obtained polypeptides were subjected to SDS-polyacrylamide gel electrophoresis and a single band for the desired G-CSF polypeptide was confirmed. The so obtained polypeptide showed human G-CSF activity. The G-CSF polypeptide was analyzed by an amino acid analyzing method with a Hitachi 835 Automatic Amino Acid Analyzer (Hitachi, Ltd.) For analysis of the N-terminal amino acids, a gas-phase sequencer (for Edman decomposition), high performance liquid chromatographic apparatus and Ultrasphere-  
50 ODS column were used.

Referential Example 10: Construction of Recombinant Vectors for Animal Cells

Recombinant vectors (derived from BPV) for use with C127 and NIH3T3 cells as host cells were  
55 constructed for each of the +VSE and -VSE line cDNAs. Recombinant vectors (with dhfr) for use with CHO cells were also constructed for each of the +VSE and -VSE line cDNAs. In the following, representative examples are described and, for further details, reference should be made to the relevant Japanese Patent Applications No. 269456/1986 and No. 270839/1986.

## (A) Construction of recombinant vectors of the +VSE line

The cDNA (+VSE) fragment obtained in Referential Example 6 was inserted into a vector pDKCR to make a plasmid pHGA410, which was partially digested with EcoRI followed by treatment with DNA polymerase I (Klenow fragment) to create blunt ends. A linker HindIII was attached to the DNA, which was subsequently treated with HindIII and T4DNA ligase. The treated DNA was used to transform *E. coli* strain DH1 by the rubidium chloride procedure (see Molecular Cloning, *ibid.*) The resulting plasmid was named pHGA410(H) (Fig. 4).

The pHGA410(H) was treated with Sall and, after blunt ends were created, it was treated with HindIII once again and a HindIII-Sall fragment was recovered. A plasmid pDBPV-1 having a transformed fragment of bovine papilloma virus was treated with HindIII and PvuII and the larger DNA fragment was separated and joined to the separately prepared HindIII-Sall fragment. The joined fragments were used to transform *E. coli* strain DH1 to obtain a plasmid, pTN-G4, which had the pHGA410-derived CSF-cDNA (Fig. 4).

Either plasmid, pHGA410 or pHGA410(H), in combination with the plasmid pAdD26SVpA was used to construct pHGG4-dhfr which was a recombinant vector (+VSE) for use with CHO cells (Fig. 5).

## (B) construction of -VSE line recombinant vectors

The cDNA (-VSE) fragment obtained in Referential Example 6 was inserted into a vector pDKCR to make a plasmid pHGV2, which was partially digested with EcoRI followed by treatment with DNA polymerase I (Klenow fragment) to create blunt ends. A linker HindIII was attached to the DNA, which was subsequently treated with HindIII and T4DNA ligase. The treated DNA was used to transform *E. coli* strain DH1 by the rubidium chloride procedure (see Molecular Cloning, *ibid.*) The resulting plasmid was named pHGV2(H) (Fig. 6).

The pHGV2(H) was treated with Sall and, after blunt ends were created, it was treated with HindIII once again and a HindIII-Sall fragment was recovered. A plasmid pDBPV-1 having transformed fragment of bovine papilloma virus was treated with HindIII and PvuII and the larger DNA fragment was separated and joined to the separately prepared HindIII-Sall fragment. The joined fragments were used to transform *E. coli* strain DH1 to obtain a plasmid, pTN-V2, which had the pHGV2-derived CSF-cDNA (Fig. 6).

By similar procedures either plasmid, pHGV2 or pHGV2(H), in combination with the plasmid pAdD26SVpA was used to construct pHGV2-dhfr which was a recombinant vector (-VSE) for use with CHO cells (Fig. 7).

Referential Example 11: Transformation of Animal Cells and G-CSF Expression Therein

Two representative examples are hereunder described for (A) the transformation of mouse C127 cells and G-CSF (+VSE) expression therein; and (B) the transformation of CHO cells and G-CSF (-VSE) expression therein.

## 40 (A) Transformation of mouse C127 cells and G-CSF (+VSE) expression therein

Before it was used to transform mouse C127 cells, the pTN-G4 obtained in Referential Example 10 (A) was treated with a restriction enzyme, BamHI. Twenty micrograms of the plasmid pTN-G4 was dissolved in 100  $\mu$ l of a reaction solution [10 mM Tris-HCl (pH 8.0), 7 mM MgCl<sub>2</sub>, 100 mM NaCl, 2 mM 2-mercaptoethanol and 0.01% BSA] and treated with 20 units of BamHI (Takara Shuzo Co., Ltd.), followed by treatments with phenol and ether, and precipitation with ethanol.

Mouse C127 cells were grown in a Dulbecco's minimal essential medium containing 10% bovine fetal serum (Gibco). The C127 cells growing on plates (5 cm<sup>2</sup>) were transformed with 10  $\mu$ g, per plate, of the separately prepared DNA by the calcium phosphate procedure [see Haynes, J. & Weissmann, C., *Nucleic Acids Res.*, 11, 687-706 (1983)]. After treatment with glycerol, the cells were incubated at 37 °C for 12 hours.

The incubated cells were transferred onto three fresh plates (5 cm<sup>2</sup>) and the media were changed twice a week. At day 16, the foci were transferred onto fresh plates and subjected to serial cultivation on a Dulbecco's minimal essential medium containing 10% bovine fetal serum (Gibco), so as to select clones having high G-CSF production rate. These clones produced G-CSF at a level of approximately 1 mg/L.

## (B) Transformation of CHO cells and G-CSF (-VSE) expression therein

CHO cells (dhfr<sup>-</sup> strain; courtesy of Dr. L. Chasin of Columbia University) were cultivated for growth in alpha-minimal essential medium containing 10% calf serum ( $\alpha$ -MEN supplemented with adenosine, deoxyadenosine and thymidine) in plates (9 cm<sup>2</sup>, Nunc). The cultured cells were transformed by the calcium phosphate procedure [Wigler et al., Cell, 14, 725 (1978)] in the following manner.

5 A carrier DNA (calf thymus DNA) was added in an appropriate amount to 1  $\mu$ g of the plasmid pHGV2-dhfr prepared in Referential Example 10 (B), and the mixture was dissolved in 375  $\mu$ l of a TE solution, followed by addition of 125  $\mu$ l of 1 M CaCl<sub>2</sub>. After the solution was cooled on ice for 3 - 5 minutes, 500  $\mu$ l of 2 x HBS (50 mM Hepes, 280 mM NaCl, and 1.5 mM phosphate buffer) was added to the solution. After re-cooling on ice, the solution was mixed with 1 ml of the culture of CHO cells, transferred onto plates, and  
10 incubated for 9 hours in a CO<sub>2</sub> incubator. The medium was removed from the plate and, following washing with TBS (Tris-buffered saline), addition of 20% glycerol-containing TBS, and re-washing, a non-selective medium (the  $\alpha$ -MEN medium described above except that it was supplemented with nucleotides) was added. After 2-day incubation, a 10-fold dilution of the culture was transferred onto a selective medium (not supplemented with nucleotides). The cultivation was continued, with the medium being replaced by a fresh  
15 selective medium every 2 days, and the resulting colonies were selected and transferred onto fresh plates, where the cells grew in the presence of 0.02  $\mu$ M methotrexate (MTX), followed by cloning through growth in the presence of 0.05  $\mu$ M MTX, which was later increased to 0.1  $\mu$ M.

The transformation of CHO cells may also be accomplished by cotransformation with pHGV2 and pAdD26SVpA [see Scahill et al., Proc. Natl. Acad. Sci., USA, 80, 4654-4658 (1983)].

20 A recombinant vector that harbors a "polycistronic gene" may also be used to transform CHO cells. An example of this alternative method is as follows: pAdD26SVpA was treated with PstI and the recovered two fragments were joined to a pBRV2-derived CSF cDNA fragment so as to construct a recombinant vector wherein the adeno virus promoter, CSF cDNA, DHFR and the poly(A) site of SV40 were inserted in the order written. This recombinant vector was used to transform CHO cells.

25 Referential Example 12: Assay of G-CSF Activity (+ VSE and -VSE)

The supernatants of cultures of C127 cells and CHO cells which were obtained in Referential Examples 11 (A) and 11 (B), respectively, were adjusted to a pH of 4 with 1 N acetic acid. After addition of an equal  
30 volume of n-propanol, the resulting precipitate was removed by centrifugation. The supernatant was passed through an open column (1<sup>st</sup> x 2 cm<sup>2</sup>) filled with a C8 reverse-phased carrier (Yamamura Kagaku K.K.) and elution was conducted with 50% n-propanol. The eluate was diluted two-fold with water and subjected to reverse-phased high performance liquid chromatography on YMC-C8 column (Yamamura Kagaku K.K.), followed by elution with n-propanol (30 - 60% linear density gradient) containing 0.1% TFA. The fractions  
35 which were eluted at n-propanol concentrations of about 40% were recovered, freeze-dried and dissolved in 0.1 M glycine buffer (pH 9). As a result of these procedures, the human G-CSF in the C127 and CHO cells was concentrated about 20-fold.

As controls, cells were transformed with human G-CSF cDNA-free plasmids and the supernatants of their cultures were concentrated in accordance with the procedures described above. The human G-CSF  
40 activities of the samples were assayed by the method of human G-CSF activity assay (a) described earlier in this specification. If the efficiency of expression is adequately high, the supernatants of cultures may be directly assayed without being concentrated. The results are summarized in Table 2, wherein the data are based on concentrated samples.

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Table 2

| Assay of Human G-CSF Activity   |   |
|---|---|
|   | Human neutrophilic colonies (colonies/dish) |
| Purified human G-CSF (20 ng)  | 96  |
| Culture of C127 cells transformed with pdBPV-1 (concentrated 20-fold)   | 0   |
| Culture of C127 cells transformed with pTNG4 (concentrated 20-fold)     | 82  |
| Culture of CHO cells transformed with pAdD26SVpA (concentrated 20-fold) | 0   |
| Culture of CHO cells transformed with pHGV2-dhfr (concentrated 20-fold) | 111   |

Referential Example 13: Amino Acid Analysis and Sugar Analysis (+VSE and -VSE)

1) Analysis of amino acid composition

The crude CSF samples prepared in Referential Example 12 were purified in accordance with the procedures described in Referential Example 2, (2), (iii). The purified CSF samples were subjected to analysis of amino acid composition by the same procedures and under the same conditions as those described in Referential Example 2, (2), (v). The results are shown in Table 3-(1) (+VSE) and Table 3-(2) (-VSE).

Table 3-(1)

| Amino Acid Analysis Data (+VSE) |       |
|---------------------------------|-------|
| Amino acids                     | Mole% |
| Asp (Asp + Asn)                 | 2.3   |
| Thr                             | 3.9   |
| Ser                             | 8.5   |
| Glu (Glu + Gln)                 | 15.3  |
| Pro                             | 7.4   |
| Gly                             | 7.8   |
| Ala                             | 10.8  |
| 1/2 Cys                         | 2.8   |
| Val                             | 4.5   |
| Met                             | 1.7   |
| Ile                             | 2.3   |
| Leu                             | 18.6  |
| Tyr                             | 1.7   |
| Phe                             | 3.4   |
| Lys                             | 2.3   |
| His                             | 2.8   |
| Trp                             | 1.1   |
| Arg                             | 2.8   |



Table 3-(2)

| Amino Acid Analysis Data (-VSE) |       |
|---------------------------------|-------|
| Amino acids                     | Mole% |
| Asp (Asp + Asn)                 | 2.3   |
| Thr                             | 4.0   |
| Ser                             | 8.1   |
| Glu (Glu + Gln)                 | 15.1  |
| Pro                             | 7.5   |
| Gly                             | 8.0   |
| Ala                             | 10.9  |
| 1/2 Cys                         | 2.8   |
| Val                             | 3.9   |
| Met                             | 1.7   |
| Ile                             | 2.3   |
| Leu                             | 18.9  |
| Tyr                             | 1.7   |
| Phe                             | 3.5   |
| Lys                             | 2.3   |
| His                             | 2.9   |
| Trp                             | 1.2   |
| Arg                             | 2.9   |

## 2) Sugar composition analysis

An internal standard (25 nmol of inositol) was added to 200 ng of each of the purified CSF samples used in the analysis of amino acid composition 1). After addition of a methanol solution (500  $\mu$ l) containing 1.5 N HCl, reaction was carried out at 90 °C for 4 hours in a N<sub>2</sub> purged, closed tube. After the tube was opened, silver carbonate (Ag<sub>2</sub>CO<sub>3</sub>) was added to neutralize the contents. Thereafter, 50  $\mu$ l of acetic anhydride was added and the tube was shaken for an adequate period. Subsequently, the tube was left overnight in the dark at room temperature. The upper layer was put into a sample tube and dried with a nitrogen gas. Methanol was added to the precipitate and the mixture was washed and lightly centrifuged. The upper layer was put into the same sample tube and dried. After addition of 50  $\mu$ l of a TMS reagent (5:1:1 mixture of pyridine, hexamethyl disilazane and trimethylchlorosilane), reaction was carried out at 40 °C for 20 minutes and the reaction product was stored in a deep freezer. A standard was prepared by combining 25 nmol of inositol with 50 nmol each of galactose (Gal), N-acetyl galactosamine (Gal NAc), sialic acid and any other appropriate reagents.

The samples thus prepared were subjected to gas chromatographic analysis under the following conditions:

### Conditions of analysis

Column : 2% OV - 17 VINport HP, 60 - 80 mesh, 3 m, glass  
 Temperature : elevated from 110 to 250 °C at 4 °C/min.  
 Carrier gas (N<sub>2</sub>) pressure : initially 1.2 - 1.6 kg/cm<sup>2</sup>  
 finally 2 - 2.5 kg/cm<sup>2</sup>  
 Sensitivity : 10<sup>3</sup> M $\Omega$  range, 0.1 - 0.4 volts  
 Pressure : H<sub>2</sub>, 0.8 kg/cm<sup>2</sup>  
 air, 0.8 kg/cm<sup>2</sup>  
 Sample feed : 2.5 - 3.0  $\mu$ l.

As a result of the analysis, galactose, N-acetyl galactosamine and sialic acid, were identified in each of the CSF samples of the present invention.

## 3) Analysis of sugar content

Each of the CSF samples used in the analysis of sugar composition in 2) was subjected to quantitative analysis of amino sugar by the Elson-Morgan method, quantitative analysis of neutral sugar by the orcinol-sulfuric acid method and quantitative analysis of sialic acid by the thiobarbituric acid method.

The procedures of the above analyses are described in Seikagaku Jikken Koza (Biochemical Experiment) Vol. 4, "Chemistry of Sugars (II)", Chap. 13 (Tokyo Kagaku Dojin).

Sugar content (weight %) of each of the samples was calculated on the basis of the measured data; the sugar content of the obtained G-CSF ranged from 1 to 20 wt% depending upon the host cell, the expression vector and the culture conditions.

10 Referential Example 14: Capability of Human G-CSF of the Present Invention to Increase the Number of Neutrophiles

Forty 8-week old male C57BL/6N mice were divided into two groups each consisting of 20 animals. One group (control group) was injected subcutaneously with 0.1 ml of a physiological saline solution containing n-propanol at a final concentration of 1% and C57BL/6N mouse serum at a final concentration of 10%. The other group (CSF treatment group) was injected subcutaneously with 0.1 ml of a physiological saline solution containing n-propanol at a final concentration of 1%, C57BL/6N mouse serum at a final concentration of 10% and 2.5 µg of the human G-CSF obtained in Referential Example 2. For each group, the injection was continued on a one-dose-a-day basis until blood sampling was conducted.

At day 2, 5, 8, 11 and 14 after the start of the injection, 4 mice were randomly sampled from each group and blood was taken from their supraorbital veins for counting the number of leukocytes with a microcell counter, Model CC180 (Toa K.K.) Blood smears were prepared and Giemsa staining was conducted in order to determine the proportion of neutrophiles in 200 leukocytes under the microscope. The number of neutrophiles in each group was calculated by the following formula:

Neutrophile counts in 1 mm<sup>3</sup> = leukocyte counts in 1 mm<sup>3</sup> x proportion of neutrophiles in leukocytes.

A group of four C57BL/6N mice to which no injection was given at all were treated by the same procedures to obtain the values for day 0. The results are shown in Table 4.

The same test was conducted except that the human G-CSF was replaced by the pure samples for amino acid analysis that were used in Referential Example 13 (+VSE derived from C127 cells and -VSE derived from CHO cells). The results were identical to those shown in Table 4.

Table 4

| Days after injection | Control group (cells/mm <sup>3</sup> ) | CSF-treated group (cells/mm <sup>3</sup> ) |
|----------------------|--|--|
| 0                    | 765 ± 139                              | 765 ± 139                                  |
| 2                    | 1344 ± 389                             | 3205 ± 439                                 |
| 5                    | 1378 ± 474                             | 4913 ± 530**                               |
| 8                    | 1127 ± 242                             | 3338 ± 310**                               |
| 11                   | 965 ± 231                              | 5229 ± 550***                              |
| 14                   | 1311 ± 197                             | 5359 ± 584***                              |

(Means ± standard error)

\*\*P<0.01,

\*\*\*P<0.001

50 Referential Example 15: Capability of Human G-CSF of the Present Invention to Inhibit Decrease in the Number of Leukocytes

Thirty-two 8-week old male ICR mice were injected peritoneally with 200 mg of cyclophosphamide (hereunder abbreviated as CP) per kg of mouse. The mice were then divided into two groups each consisting of 16 animals. One group (control group) was injected subcutaneously with 0.1 ml of a physiological saline solution containing n-propanol at a final concentration of 1% and IRC mouse serum at a final concentration of 10%. The other group (CSF treatment group) was injected subcutaneously with 0.1 ml of a physiological saline solution containing n-propanol at a final concentration of 1%, ICR mouse serum at a final concentration of 10%, and 2.5 µg of the human G-CSF obtained in Referential Example 2. For each

group, the injection was continued on a one-dose-a-day basis until blood sampling was conducted.

At day 2, 4, 5 and 7 after the start of the CP injection, 4 mice were randomly sampled from each group and blood was taken from their supraorbital veins so that the number of leukocytes could be counted with a microcell counter, Model CC180 (Toa K.K.). Blood smears were prepared and Giemsa staining was conducted in order to determine the proportion of neutrophils in 200 leukocytes under the microscope. The number of neutrophils in each group was calculated by the following formula:

Neutrophile counts in  $1 \text{ mm}^3$  = leukocyte counts in  $1 \text{ mm}^3$  x proportion of neutrophils in leukocytes.

The stained neutrophils were classified morphologically in order to determine the proportion of segmented neutrophils which were the most mature form of neutrophils.

A group of four C57BL/6N mice to which no injection was given at all were treated by the same procedures to obtain the values for day 0. The results are shown in Table 5. The same test was conducted employing the pure samples of human G-CSF used in amino acid analysis in Referential Example 13 (+VSE derived from C127 cells and -VSE from CHO cells). The results were identical to those shown in Table 5.

Table 5

| Control group       |  |  |   |
|---------------------|--|--|---|
| Days                | leukocyte count<br>(cell/mm <sup>3</sup> ) | neutrophile count<br>(cell/mm <sup>3</sup> ) | proportion of<br>segmented<br>neutrophils (%) |
| 0                   | 7800 ± 297                                 | 1068 ± 131                                   | 84 ± 4  |
| 2                   | 2200 ± 187                                 | 866 ± 131                                    | 91 ± 3  |
| 4                   | 1650 ± 132                                 | 59 ± 4                                       | 29 ± 14                                       |
| 5                   | 2050 ± 253                                 | 236 ± 36                                     | 28 ± 4  |
| 7                   | 3775 ± 551                                 | 1042 ± 462                                   | 38 ± 5  |
| CSF treatment group |  |  |   |
| Days                | leukocyte count<br>(cell/mm <sup>3</sup> ) | neutrophile count<br>(cell/mm <sup>3</sup> ) | proportion of<br>segmented<br>neutrophils (%) |
| 0                   | 7800 ± 297                                 | 1068 ± 131                                   | 84 ± 4  |
| 2                   | 3750 ± 724                                 | 2417 ± 708                                   | 98 ± 1  |
| 4                   | 3975 ± 103***                              | 1492 ± 129***                                | 54 ± 6***                                     |
| 5                   | 7100 ± 1127**                              | 5017 ± 959**                                 | 89 ± 7**                                      |
| 7                   | 3725 ± 347                                 | 965 ± 175                                    | 56 ± 5  |

(Mean ± standard error) \*\*p<0.01, \*\*\*p<0.001

As Table 4 shows, no increase in the neutrophile count was observed in the control group of normal animals but, in the CSF-treated group, a significant increase in the neutrophile count was observed at day 2 after the administration of CSF and a gradual increase occurred thereafter so long as the CSF supply was continued. As is clear from Table 5 showing the results for animal models with neutropenia, the decrease in the neutrophile count was continued in the control group until day 5 after the CP administration but, in the CSF treated group, the neutrophile counts observed for period of 7 days after the CP administration were

equal to or higher than the initial values.

The improvement achieved by the administration of human G-CSF was apparent not only for the absolute number of peripheral neutrophils but also with respect to their maturity. The decrease in the number of peripheral leukocytes was also mitigated by the administration of human G-CSF.

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Referential Example 16: Effect of human G-CSF on leukopenic models

10 Thirty-six C57BL/6N mice (male, 8-week old) were injected intraperitoneally with 200 mg/kg of CP. Four days later, 4 mice were randomly sampled and blood was taken from their supraorbital veins so that it could be verified that the numbers of peripheral leukocytes and neutrophils had been adequately lowered. The remaining 32 mice were divided into two groups each consisting of 16 animals. Starting on the next day, one group (control group) was injected subcutaneously with 0.1 ml of a physiological saline solution containing n-propranolol at a final concentration of 1% and C57BL/6N mouse serum at a final concentration of 10%, and the other group (CSF treatment group) was injected subcutaneously with 0.1 ml of a physiological saline solution containing n-propranolol at a final concentration of 1%, C57BL/6N mouse serum at a final concentration of 10%, and 2.5 µg of the human G-CSF obtained in Referential Example 2. For each group, the injection was continued for 2 days on a one-dose-a-day basis. On predetermined days, 4 mice were randomly sampled from each group and blood was taken from their supraorbital veins so that the numbers of leukocytes and neutrophils could be determined. In this experiment, all administrations were conducted at 9 o'clock a.m. and all blood samples were taken at 3 p.m. Counting of the leukocytes in the blood samples, their classification and the determination of the number of peripheral neutrophils were conducted as in Referential Example 15. A group of four C57BL/6N mice to which no injection was given at all were treated by the same procedure to obtain the values for day 0. In this experiment, only one blood sample was taken from each mouse.

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25 The results of the experiment are shown in Table 6. The same experiment was repeated using the pure samples of human G-CSF that were employed for amino acid assaying in Referential Example 13 (+VSE derived from C127 cells and -VSE from CHO cells). The results obtained were identical to those shown in Table 6.

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Table 6

| Peripheral leukocyte count (cell/mm <sup>3</sup> )   |               |                   |
|--|---------------|-------------------|
| Days   | control group | CSF treated group |
| 0  | 9864 ± 1121   |                   |
| 4  | 1577 ± 266    |                   |
| 5  | 3061 ± 374    | 7946 ± 504***     |
| 6  | 4866 ± 701    | 29113 ± 1544***   |
| 7  | 8212 ± 1824   | 8300 ± 3210       |
| 11   | 10207 ± 984   | 11544 ± 844       |
| Peripheral neutrophile count (cell/mm <sup>3</sup> ) |               |                   |
| Days   | control group | CSF treated group |
| 0  | 1162 ± 104    |                   |
| 4  | 68 ± 7        |                   |
| 5  | 236 ± 36      | 1208 ± 25***      |
| 6  | 483 ± 42      | 24168 ± 173***    |
| 7  | 3062 ± 462    | 5746 ± 1920       |
| 11   | 987 ± 49      | 1055 ± 105        |

(Mean ± standard error) \*\*\*p<0.001

As Table 6 shows, the administration of G-CSF induced a distinct recovery from the decrease in the numbers of leukocytes and neutrophiles.

In this connection, it should be emphasized that the aforementioned results were attained not only with the human G-CSF obtained from the supernatant of the culture of human G-CSF producing cells but also with the human G-CSF that the assignee of the present invention successfully produced from the transformants harboring the gene coding for said human G-CSF.

These results suggest the effectiveness of the human G-CSF of the present invention as a leukopenia treating agent.

It should also be mentioned that no toxicity was found under the conditions employed in the experiments described above.

#### Example 1

The human G-CSF containing fraction obtained by the method described in Referential Example 2 was rendered germ-free and frozen at -20° C. The frozen fraction was worked up to prepare an injection.

#### Example 2

The C127 cell-derived human G-CSF of +VSE line obtained in Referential Example 12 was purified by the method described in Referential Example 2, (2), (iii), aseptically charged in amounts of 5 ml in 10-ml vials, and freeze-dried at -20° C, with the vials being subsequently closed with rubber stoppers. The so

obtained freeze-dried products were worked up to prepare an injection preparation.

### Example 3

5 The CHO cell-derived human G-CSF of -VSE line obtained in Referential Example 12 was purified by the method described in Referential Example 2, (2), (iii), aseptically charged in amounts of 5 ml in 10-ml vials and freeze-dried at  $-20^{\circ}\text{C}$ , with the vials being subsequently closed with rubber stoppers. The so obtained freeze-dried products were worked up to prepare an injection preparation.

### 10 Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, IT, LI, NL, SE

1. A leukopenia treating agent containing a human granulocyte colony stimulating factor as an effective ingredient.
- 15 2. A leukopenia treating agent according to Claim 1 wherein the human granulocyte colony stimulating factor is a neutrophile colony stimulating factor.
3. A leukopenia treating agent according to Claim 1 wherein the human granulocyte colony stimulating factor is obtained from the supernatant of the culture of a human granulocyte colony stimulating factor producing cell.
- 20 4. A leukopenia treating agent according to Claim 3 wherein the human granulocyte colony stimulating factor has the following physicochemical properties:
  - 25 i) molecular weight: about  $19,000 \pm 1,000$  as measured by electrophoresis through a sodium dodecylsulfate - polyacrylamide gel;
  - ii) isoelectric point: having at least one of the three isoelectric points,  $\text{pI} = 5.5 \pm 0.1$ ,  $\text{pI} = 5.8 \pm 0.1$ , and  $\text{pI} = 6.1 \pm 0.1$ ;
  - 30 iii) ultraviolet absorption: having a maximum absorption at 280 nm and a minimum absorption at 250 nm;
  - iv) amino acid sequence of the 21 residues from N terminus:

35  $\text{H}_2\text{N} - \text{Thr} - \text{Pro} - \text{Leu} - \text{Gly} - \text{Pro} - \text{Ala} - \text{Ser} - \text{Ser} -$   
 $\text{Leu} - \text{Pro} - \text{Gln} - \text{Ser} - \text{Phe} - \text{Leu} - \text{Leu} - \text{Lys} - \text{Cys} -$   
 $\text{Leu} - \text{Glu} - \text{Gln} - \text{Val}$

- 40 5. A leukopenia treating agent according to Claim 1 wherein the human granulocyte colony stimulating factor is a polypeptide or glycoprotein having the human granulocyte colony stimulating factor activity which is produced from a transformant containing a recombinant vector harboring a gene coding for the polypeptide having the human granulocyte colony stimulating factor activity.
- 45 6. A leukopenia treating agent according to Claim 5 wherein the gene coding for the polypeptide having the human granulocyte colony stimulating factor activity has all or part of the nucleotide sequence shown in accompanying Fig. 2.
7. A leukopenia treating agent according to Claim 5 wherein the gene coding for the polypeptide having the human granulocyte colony stimulating factor activity has all or part of the nucleotide sequence shown in accompanying Fig. 3.
- 50 8. A leukopenia treating agent according to Claim 5 wherein the polypeptide having the human granulocyte colony stimulating factor activity is represented by all or part of the amino acid sequence shown below:
- 55

5 (Met)<sub>n</sub> Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro  
 Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val  
 Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
 Glu Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys  
 Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly  
 10 His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser  
 Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly  
 Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu  
 Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile  
 15 Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu  
 Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile  
 Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro  
 20 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala  
 Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu  
 25 Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala  
 Gln Pro

(provided that m is 0 or 1; and n is 0 or 1).

- 30 9. A leukemia treating agent according to Claim 5 wherein the glycoprotein having the human granulocyte colony stimulating factor activity has a polypeptide and a sugar chain portion, said polypeptide being represented by all or part of the amino acid sequence shown below:

35 Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln  
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg  
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 40 Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys Leu  
 Cys His Pro Glu Glu Leu Val Leu Leu Gly His

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Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser  
 Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys  
 5 Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr  
 Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser  
 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln  
 10 Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe  
 15 Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val  
 Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu  
 Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln  
 Pro

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(provided that m is 0 or 1).

**Claims for the following Contracting States : AT, ES**

- 25 1. A method for preparing leukopenia treating agent which comprises combining a human granulocyte colony stimulating factor as effective ingredient with a pharmaceutically acceptable carrier or excipient and optionally a stabilizer and/or anti-adsorption agent.
- 30 2. The method according to Claim 1 wherein the human granulocyte colony stimulating factor is a neutrophile colony stimulating factor.
- 35 3. The method according to Claim 1 wherein the human granulocyte colony stimulating factor is obtained from the supernatant of the culture of a human granulocyte colony stimulating factor producing cell.
- 40 4. The method according to Claim 3 wherein the human granulocyte colony stimulating factor has the following physicochemical properties:
- i) molecular weight: about  $19,000 \pm 1,000$  as measured by electrophoresis through a sodium dodecylsulfate - polyacrylamide gel;
  - ii) isoelectric point: having at least one of the three isoelectric points,  $pI = 5.5 \pm 0.1$ ,  $pI = 5.8 \pm 0.1$ , and  $pI = 6.1 \pm 0.1$ ;
  - iii) ultraviolet absorption: having a maximum absorption at 280 nm and a minimum absorption at 250 nm;
  - iv) amino acid sequence of the 21 residues from N terminus:

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$H_2N$  - Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser -  
 Leu - Pro - Gln - Ser - Phe - Leu - Leu - Lys - Cys -  
 50 Leu - Glu - Gln - Val

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- 55 5. The method according to Claim 1 wherein the human granulocyte colony stimulating factor is a polypeptide or glycoprotein having the human granulocyte colony stimulating factor activity which is produced from a transformant containing a recombinant vector harboring a gene coding for the polypeptide having the human granulocyte colony stimulating factor activity.



6. The method according to Claim 5  
 wherein the gene coding for the polypeptide having the human granulocyte colony stimulating factor activity has all or part of the nucleotide sequence shown in accompanying Fig. 2.
- 5 7. The method according to Claim 5  
 wherein the gene coding for the polypeptide having the human granulocyte colony stimulating factor activity has all or part of the nucleotide sequence shown in accompanying Fig. 3.
8. The method according to Claim 5  
 10 wherein the polypeptide having the human granulocyte colony stimulating factor activity is represented by all or part of the amino acid sequence shown below:

|    |                    |     |     |      |     |                   |     |     |     |     |     |
|----|--------------------|-----|-----|------|-----|-------------------|-----|-----|-----|-----|-----|
|    | (Met) <sub>n</sub> | Thr | Pro | Leu  | Gly | Pro               | Ala | Ser | Ser | Leu | Pro |
| 15 | Gln                | Ser | Phe | Leu  | Leu | Lys               | Cys | Leu | Glu | Gln | Val |
|    | Arg                | Lys | Ile | Gln  | Gly | Asp               | Gly | Ala | Ala | Leu | Gln |
|    | Glu                | Lys | Leu | (Val | Ser | Glu) <sub>m</sub> | Cys | Ala | Thr | Tyr | Lys |
| 20 | Leu                | Cys | His | Pro  | Glu | Glu               | Leu | Val | Leu | Leu | Gly |
|    | His                | Ser | Leu | Gly  | Ile | Pro               | Trp | Ala | Pro | Leu | Ser |
|    | Ser                | Cys | Pro | Ser  | Gln | Ala               | Leu | Gln | Leu | Ala | Gly |
|    | Cys                | Leu | Ser | Gln  | Leu | His               | Ser | Gly | Leu | Phe | Leu |
| 25 | Tyr                | Gln | Gly | Leu  | Leu | Gln               | Ala | Leu | Glu | Gly | Ile |
|    | Ser                | Pro | Glu | Leu  | Gly | Pro               | Thr | Leu | Asp | Thr | Leu |
|    | Gln                | Leu | Asp | Val  | Ala | Asp               | Phe | Ala | Thr | Thr | Ile |
| 30 | Trp                | Gln | Gln | Met  | Glu | Glu               | Leu | Gly | Met | Ala | Pro |
|    | Ala                | Leu | Gln | Pro  | Thr | Gln               | Gly | Ala | Met | Pro | Ala |
|    | Phe                | Ala | Ser | Ala  | Phe | Gln               | Arg | Arg | Ala | Gly | Gly |
| 35 | Val                | Leu | Val | Ala  | Ser | His               | Leu | Gln | Ser | Phe | Leu |
|    | Glu                | Val | Ser | Tyr  | Arg | Val               | Leu | Arg | His | Leu | Ala |
|    | Gln                | Pro |     |      |     |                   |     |     |     |     |     |

40 (provided that m is 0 or 1; and n is 0 or 1).

9. The method according to Claim 5  
 wherein the glycoprotein having the human granulocyte colony stimulating factor activity has a polypeptide and a sugar chain portion, said polypeptide being represented by all or part of the amino acid sequence shown below:
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Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln  
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg  
 5 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys Leu  
 Cys His Pro Glu Glu Leu Val Leu Leu Gly His  
 10 Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser  
 Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys  
 Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr  
 Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser  
 15 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln  
 Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 20 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe  
 Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val  
 Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu  
 Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln  
 25 Pro

(provided that m is 0 or 1).

30 **Revendications**

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, IT, LI, NL, SE

1. Agent de traitement de la leucopénie contenant un facteur de stimulation de colonie de granulocytes humains comme constituant efficace.
- 35 2. Agent de traitement de la leucopénie suivant la revendication 1, dans lequel le facteur de stimulation de colonie de granulocytes humains est un facteur de stimulation de colonie de neutrophiles.
- 40 3. Agent de traitement de la leucopénie suivant la revendication 1, dans lequel le facteur de stimulation de colonie de granulocytes humains est obtenu à partir du surnageant de la culture d'une cellule produisant le facteur de stimulation de colonie de granulocytes humains.
- 45 4. Agent de traitement de la leucopénie suivant la revendication 3, dans lequel le facteur de stimulation de colonie de granulocytes humains a les propriétés physico-chimiques suivantes :
  - i) poids moléculaire :  
environ  $19\ 000 \pm 1\ 000$  tel que mesuré par électrophorèse dans un gel de polyacrylamide avec dodécylsulfate de sodium;
  - ii) point isoélectrique :  
ayant au moins l'un des trois points isoélectriques,  $pI = 5,5 \pm 0,1$ ,  $pI = 5,8 \pm 0,1$  et  $pI = 6,1 \pm 0,1$ ;
  - 50 iii) absorption dans l'ultraviolet :  
ayant un maximum d'absorption à 280 nm et un minimum d'absorption à 250 nm;
  - iv) séquence d'acides aminés des 21 résidus depuis l'extrémité N-terminale :

55 H<sub>2</sub>N - Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser -  
 Leu - Pro - Gln - Ser - Phe - Leu - Leu - Lys - Cys -  
 Leu - Glu - Gln - Val.

5. Agent de traitement de la leucopénie suivant la revendication 1, dans lequel le facteur de stimulation de colonie de granulocytes humains est un polypeptide ou une glycoprotéine ayant l'activité de facteur de stimulation de colonie de granulocytes humains qui est produit par un transformant contenant un vecteur recombinant hébergeant un gène codant pour le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains.
6. Agent de traitement de la leucopénie suivant la revendication 5, dans lequel le gène codant pour le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains a tout ou partie de la séquence de nucléotides représentée à la Fig. 2 annexée.
7. Agent de traitement de la leucopénie suivant la revendication 5, dans lequel le gène codant pour le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains a tout ou partie de la séquence de nucléotides représentée à la Fig. 3 annexée.
8. Agent de traitement de la leucopénie suivant la revendication 5, dans lequel le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains est représenté par tout ou partie de la séquence d'acides aminés indiquée ci-après :

(Met)<sub>n</sub> Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro  
 Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val  
 Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
 Glu Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys  
 Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly  
 His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser  
 Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly  
 Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu  
 Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile  
 Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu  
 Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile  
 Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro

Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala  
 Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu  
 Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala  
 Gln Pro

(avec la restriction que m est 0 ou 1 et n est 0 ou 1).

9. Agent de traitement de la leucopénie suivant la revendication 5, dans lequel la glycoprotéine ayant l'activité de facteur de stimulation de colonie de granulocytes humains comprend un polypeptide et une partie chaîne de sucre, le polypeptide étant représenté par tout ou partie de la séquence d'acides aminés indiquée ci-après :

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln  
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg  
 5 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys Leu  
 Cys His Pro Glu Glu Leu Val Leu Leu Gly His  
 10 Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser  
 Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys  
 Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr  
 15 Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser  
 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln  
 Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 20 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe  
 Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val  
 Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu  
 25 Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln  
 Pro,

(avec la restriction que m est 0 ou 1).

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Revendications pour les Etats contractants suivants : AT, ES

1. Procédé de préparation d'un agent de traitement de la leucopénie qui comprend la combinaison d'un facteur de stimulation de colonie de granulocytes humains comme constituant actif avec un excipient ou support pharmaceutiquement acceptable et facultativement un stabilisant et/ou un agent antiadsorption.
2. Procédé suivant la revendication 1, dans lequel le facteur de stimulation de colonie de granulocytes humains est un facteur de stimulation de colonie de neutrophiles.
3. Procédé suivant la revendication 1, dans lequel le facteur de stimulation de colonie de granulocytes humains est obtenu à partir du surnageant de la culture d'une cellule produisant le facteur de stimulation de colonie de granulocytes humains.
4. Procédé suivant la revendication 3, dans lequel le facteur de stimulation de colonie de granulocytes humains a les propriétés physico-chimiques suivantes :
  - i) poids moléculaire : environ  $19\ 000 \pm 1\ 000$  tel que mesuré par électrophorèse dans un gel de Polyacrylamide avec dodécylsulfate de sodium;
  - ii) point isoélectrique : ayant au moins l'un des trois points isoélectriques,  $pI = 5,5 \pm 0,1$ ,  $pI = 5,8 \pm 0,1$  et  $pI = 6,1 \pm 0,1$ ;
  - iii) absorption dans l'ultraviolet : ayant un maximum d'absorption à 280 nm et un minimum d'absorption à 250 nm;
  - iv) séquence d'acides aminés des 21 résidus depuis l'extrémité N terminale :

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H<sub>2</sub>N - Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser -  
 Leu - Pro - Gln - Ser - Phe - Leu - Leu - Lys - Cys -  
 Leu - Glu - Gln - Val.

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5. Procédé suivant la revendication 1, dans lequel le facteur de stimulation de colonie de granulocytes humains est un polypeptide ou une glycoprotéine ayant l'activité de facteur de stimulation de colonie de granulocytes humains qui est produit par un transformant contenant un vecteur recombinant hébergeant un gène codant pour le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains.

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6. Procédé suivant la revendication 5, dans lequel le gène codant pour le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains a tout ou partie de la séquence de nucléotides représentée à la Fig. 2 annexée.

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7. Procédé suivant la revendication 5, dans lequel le gène codant pour le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains a tout ou partie de la séquence de nucléotides représentée à la Fig. 3 annexée.

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8. Procédé suivant la revendication 5, dans lequel le polypeptide ayant l'activité de facteur de stimulation de colonie de granulocytes humains est représenté par tout ou partie de la séquence d'acides aminés indiquée ci-après :

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(Met)<sub>n</sub> Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro  
 Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val  
 30 Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
 Glu Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys  
 Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly  
 35 His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser  
 Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly  
 Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu  
 Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile  
 40 Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu

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Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile  
 Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro  
 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala  
 50 Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu  
 Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala  
 Gln Pro

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(avec la restriction que m est 0 ou 1 et n est 0 ou 1).

9. Procédé suivant la revendication 5, dans lequel la glycoprotéine ayant l'activité de facteur de stimulation de colonie de granulocytes humains comprend un polypeptide et une partie chaîne de sucre, le polypeptide étant représenté par tout ou partie de la séquence d'acides aminés indiquée ci-après :

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Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln  
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg  
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys Leu  
 Cys His Pro Glu Glu Leu Val Leu Leu Gly His  
 Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser  
 Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys  
 Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr  
 Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser  
 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln  
 Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe  
 Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val  
 Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu  
 Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln  
 Pro,

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(avec la restriction que m est 0 ou 1).

**Patentansprüche**

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, IT, LI, NL, SE

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1. Wirkstoff zur Behandlung von Leukopenie, enthaltend einen menschlichen Granulocyten-Kolonie-stimulierenden Faktor als wirksamen Bestandteil.

2. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 1, wobei der menschliche Granulocyten-Kolonie-stimulierende Faktor ein Neutrophilen-Kolonie-stimulierender Faktor ist.

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3. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 1, wobei man den menschlichen Granulocyten-Koloniestimulierenden Faktor aus dem Kulturüberstand einer menschlichen Granulocyten-Kolonie-stimulierenden Faktor herstellenden Zelle erhält.

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4. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 3, wobei der menschliche Granulocyten-Kolonie-stimulierende Faktor die nachfolgenden physikalisch-chemischen Eigenschaften aufweist:

i) Molekulargewicht:

etwa 19 000 ± 1000 nach Bestimmung durch Elektrophorese in einem Natriumdodecylsulfat-Polyacrylamidgel;

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ii) Isoelektrischer Punkt:

Er weist mindestens einen der drei isoelektrischen Punkte  $pI = 5,5 \pm 0,1$ ,  $pI = 5,8 \pm 0,1$  und  $pI = 6,1 \pm 0,1$  auf;

iii) Ultraviolette Absorption:

Er weist eine maximale Absorption bei 280 nm und eine minimale Absorption bei 250 nm auf;  
iv) Aminosäuresequenz der 21 N-terminalen Reste:

5           H<sub>2</sub>N - Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser -  
            Leu - Pro - Gln - Ser - Phe - Leu - Leu - Lys - Cys -  
            Leu - Glu - Gln - Val

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5. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 1, wobei der menschliche Granulocyten-Kolonie-stimulierende Faktor ein Polypeptid oder Glykoprotein mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors ist, das von einer Transformante hergestellt wird, die einen rekombinanten Vektor enthält, der ein ein Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors codierendes Gen trägt.

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6. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 5, wobei das das Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors codierende Gen die gesamte oder einen Teil der in der beigefügten Figur 2 dargestellten Nucleotidsequenz aufweist.

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7. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 5, wobei das das Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors codierende Gen die gesamte oder einen Teil der in der beigefügten Figur 3 dargestellten Nucleotidsequenz aufweist.

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8. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 5, wobei das Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors durch die gesamte oder einen Teil der nachfolgend dargestellten Aminosäuresequenz verkörpert wird:

30           (Met)<sub>n</sub> Thr   Pro   Leu   Gly   Pro   Ala   Ser   Ser   Leu   Pro  
            Gln   Ser   Phe   Leu   Leu   Lys   Cys   Leu   Glu   Gln   Val  
            Arg   Lys   Ile   Gln   Gly   Asp   Gly   Ala   Ala   Leu   Gln  
            Glu   Lys   Leu   (Val   Ser   Glu)<sub>m</sub> Cys   Ala   Thr   Tyr   Lys  
35           Leu   Cys   His   Pro   Glu   Glu   Leu   Val   Leu   Leu   Gly  
            His   Ser   Leu   Gly   Ile   Pro   Trp   Ala   Pro   Leu   Ser  
            Ser   Cys   Pro   Ser   Gln   Ala   Leu   Gln   Leu   Ala   Gly  
40           Cys   Leu   Ser   Gln   Leu   His   Ser   Gly   Leu   Phe   Leu  
            Tyr   Gln   Gly   Leu   Leu   Gln   Ala   Leu   Glu   Gly   Ile  
            Ser   Pro   Glu   Leu   Gly   Pro   Thr   Leu   Asp   Thr   Leu  
            Gln   Leu   Asp   Val   Ala   Asp   Phe   Ala   Thr   Thr   Ile  
45           Trp   Gln   Gln   Met   Glu   Glu   Leu   Gly   Met   Ala   Pro  
            Ala   Leu   Gln   Pro   Thr   Gln   Gly   Ala   Met   Pro   Ala  
            Phe   Ala   Ser   Ala   Phe   Gln   Arg   Arg   Ala   Gly   Gly  
50           Val   Leu   Val   Ala   Ser   His   Leu   Gln   Ser   Phe   Leu  
            Glu   Val   Ser   Tyr   Arg   Val   Leu   Arg   His   Leu   Ala  
            Gln   Pro

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(vorausgesetzt, daß m = 0 oder 1 und n = 0 oder 1).

9. Wirkstoff zur Behandlung von Leukopenie nach Anspruch 5, wobei das Glykoprotein mit der Aktivität

des menschlichen Granulocyten-Kolonie-stimulierenden Faktors ein Polypeptid und einen Zuckerkettenanteil aufweist, wobei das Polypeptid durch die gesamte oder einen Teil der nachfolgend dargestellten Aminosäuresequenz verkörpert wird:

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Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln  
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg  
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys Leu  
 Cys His Pro Glu Glu Leu Val Leu Leu Gly His  
 Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser  
 Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys  
 Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr  
 Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser

Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln  
 Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe  
 Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val  
 Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu  
 Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln  
 Pro

(vorausgesetzt, daß  $m = 0$  oder  $1$ ).

#### Patentansprüche für folgende Vertragsstaaten : AT, ES

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1. Verfahren zur Herstellung eines Wirkstoffes zur Behandlung von Leukopenie, wobei man einen menschlichen Granulocyten-Kolonie-stimulierenden Faktor als wirksamen Bestandteil mit einem pharmazeutisch verträglichen Träger oder Exzipienten und gegebenenfalls einem Stabilisator und/oder Anti-Adsorptionswirkstoff kombiniert.
2. Verfahren nach Anspruch 1, wobei der menschliche Granulocyten-Kolonie-stimulierende Faktor ein Neutrophilen-Kolonie-stimulierender Faktor ist.
3. Verfahren nach Anspruch 1, wobei man den Granulocyten-Kolonie-stimulierenden Faktor aus dem Kulturüberstand einer menschlichen Granulocyten-Kolonie-stimulierenden Faktor herstellenden Zelle erhält.
4. Verfahren nach Anspruch 3, wobei der menschliche Granulocyten-Kolonie-stimulierende Faktor die nachfolgenden physikalisch-chemischen Eigenschaften aufweist:
  - i) Molekulargewicht:  
etwa  $19\ 000 \pm 1000$  nach Bestimmung durch Elektrophorese in einem Natriumdodecylsulfat-Polyacrylamidgel;
  - ii) Isoelektrischer Punkt:



Er weist mindestens einen der drei isoelektrischen Punkte  $pI = 5,5 \pm 0,1$ ,  $pI = 5,8 \pm 0,1$  und  $pI = 6,1 \pm 0,1$  auf;

iii) Ultraviolette Absorption:

Er weist eine maximale Absorption bei 280 nm und eine minimale Absorption bei 250 nm auf;

iv) Aminosäuresequenz der 21 N-terminalen Reste:

H<sub>2</sub>N - Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser -  
 Leu - Pro - Gln - Ser - Phe - Leu - Leu - Lys - Cys -  
 Leu - Glu - Gln - Val

5. Verfahren nach Anspruch 1, wobei der menschliche Granulocyten-Kolonie-stimulierende Faktor ein Polypeptid oder Glykoprotein mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors ist, das von einer Transformante hergestellt wird, die einen rekombinanten Vektor enthält, der ein Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors codierendes Gen trägt.
6. Verfahren nach Anspruch 5, wobei das Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors codierende Gen die gesamte oder einen Teil der in der beigefügten Figur 2 dargestellten Nucleotidsequenz aufweist.
7. Verfahren nach Anspruch 5, wobei das Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors codierende Gen die gesamte oder einen Teil der in der beigefügten Figur 3 dargestellten Nucleotidsequenz aufweist.
8. Verfahren nach Anspruch 5, wobei das Polypeptid mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors durch die gesamte oder einen Teil der nachfolgend dargestellten Aminosäuresequenz verkörpert wird:

(Met)<sub>n</sub> Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro  
 Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val  
 Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
 Glu Lys Leu (Val Ser Glu)<sub>m</sub> Cys Ala Thr Tyr Lys  
 Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly  
 His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser  
 Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly  
 Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu  
 Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile  
 Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu  
 Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile  
 Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro  
 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala  
 Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu  
 Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala  
 Gln Pro

(vorausgesetzt, daß  $m = 0$  oder  $1$  und  $n = 0$  oder  $1$ ).

9. Verfahren nach Anspruch 5, wobei das Glykoprotein mit der Aktivität des menschlichen Granulocyten-Kolonie-stimulierenden Faktors ein Polypeptid und einen Zuckerkettenanteil aufweist, wobei das Polypeptid durch die gesamte oder einen Teil der nachfolgend dargestellten Aminosäuresequenz verkörpert wird:

|    |     |     |      |     |      |                  |     |     |     |     |     |
|----|-----|-----|------|-----|------|------------------|-----|-----|-----|-----|-----|
|    | Thr | Pro | Leu  | Gly | Pro  | Ala              | Ser | Ser | Leu | Pro | Gln |
| 10 | Ser | Phe | Leu  | Leu | Lys  | Cys              | Leu | Glu | Gln | Val | Arg |
|    | Lys | Ile | Gln  | Gly | Asp  | Gly              | Ala | Ala | Leu | Gln | Glu |
|    | Lys | Leu | (Val | Ser | Glu) | <sub>m</sub> Cys | Ala | Thr | Tyr | Lys | Leu |
| 15 | Cys | His | Pro  | Glu | Glu  | Leu              | Val | Leu | Leu | Gly | His |
|    | Ser | Leu | Gly  | Ile | Pro  | Trp              | Ala | Pro | Leu | Ser | Ser |
|    | Cys | Pro | Ser  | Gln | Ala  | Leu              | Gln | Leu | Ala | Gly | Cys |
| 20 | Leu | Ser | Gln  | Leu | His  | Ser              | Gly | Leu | Phe | Leu | Tyr |
|    | Gln | Gly | Leu  | Leu | Gln  | Ala              | Leu | Glu | Gly | Ile | Ser |
|    | Pro | Glu | Leu  | Gly | Pro  | Thr              | Leu | Asp | Thr | Leu | Gln |
| 25 | Leu | Asp | Val  | Ala | Asp  | Phe              | Ala | Thr | Thr | Ile | Trp |
|    | Gln | Gln | Met  | Glu | Glu  | Leu              | Gly | Met | Ala | Pro | Ala |
| 30 | Leu | Gln | Pro  | Thr | Gln  | Gly              | Ala | Met | Pro | Ala | Phe |
|    | Ala | Ser | Ala  | Phe | Gln  | Arg              | Arg | Ala | Gly | Gly | Val |
|    | Leu | Val | Ala  | Ser | His  | Leu              | Gln | Ser | Phe | Leu | Glu |
| 35 | Val | Ser | Tyr  | Arg | Val  | Leu              | Arg | His | Leu | Ala | Gln |
|    | Pro |     |      |     |      |                  |     |     |     |     |     |

40 (vorausgesetzt, daß  $m = 0$  oder  $1$ ).

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Fig. 1

Probe (IWQ)

Ile Trp Gln Gln Met Glu Glu Leu Gly Met

5'----- ATG TGG CAA CAA ATG GAA GAA CTG GGT ATG -----3'  
                   G  G          G  G  T

Probe (A)

Met Pro Ala Phe Ala

5'----- ATG CCA GCA TTT GC -----3'  
                   T  T  C  
                   G  G  
                   C  C

3'----- TAC GGA CGA AAA CG -----5'  
                   T  T  G  
                   G  G  
                   C  C

} Probe (A)

Probe (LC)

Gln Glu Lys Leu Cys Ala Thr Tyr

5'----- CAG GAG AAG CTG TGT GCC ACC TAC -----3'

3'----- GTC CTC TTC GAC ACA CGG TGG ATG -----5' Probe (LC)

Fig. 2-1

CGGAGCCTGCAGCCCAGCCCCACCCAGACCC 10 30

ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG AAG CTG ATG GCC CTG CAG CTG CTG CTG TGG 50 90  
 Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys Leu Met Ala Leu Gln Leu Leu Leu Trp  
 -30

CAC AGT GCA CTC TGG ACA GTG CAG GAA GCC ACC CCC CTG GGC CCT GCC AGC TCC CTG CCC 110 150  
 His Ser Ala Leu Trp Thr Val Gln Glu Ala Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro  
 -10

CAG AGC TTC CTG CTC AAG TGC TTA GAG CAA GTG AAG ATC CAG GGC GAT GGC GCA GCG 170 210  
 Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala  
 20

CTC CAG GAG AAG CTG GTG AGT GAG TGT GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG 230 270  
 Leu Gln Glu Lys Leu Val Ser Glu Ser Glu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu  
 40

GTG CTG CTC GGA CAC TCT CTG GGC ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC AGC CAG 290 330  
 Val Leu Leu Gly His Ser Leu Glu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln  
 60

GCC CTG CAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC GGC CTT TTC CTC TAC CAG GGG 350 390  
 Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly  
 80 90

Fig. 2-2

410 CTC CTG CAG GCC CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC ACC TTG GAC ACA CTG CAG 450  
 Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln 110  
 100  
 470 CTG GAC GTC GCC GAC TTT GCC ACC ACC ATC TGG CAG CAG ATG GAA GAA CIG GGA ATG GCC 510  
 Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala 130  
 120  
 530 CCT GCC CTG CAG CCC ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC CGG 570  
 Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg 150  
 140  
 590 GCA GGA GGG GTC CTG GTT GCC TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG TAC CGC GTT 630  
 Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val 170  
 160  
 650 CTA CGC CAC CTT GCC CAG CCC TGA GCCAAGCCCTCCCATCCCATGATTTATCTCTATTTAATATTATG 690  
 Leu Arg His Leu Ala Gln Pro End  
 710 TCTATTTAAGCCTCATATTTAAAGACAGGGGAAGAGCAGAACGGAGCCCCAGGCCCTGTGTCCTTCCCTGCATTTCIG 750  
 730  
 790 AGTTTCATTCCTCCCTGTAGCAGTGAGAAAAGCTCCTGTCTCCCATCCCTGGACIGGGAGGTAGATAGGTAAT 830  
 810  
 870 ACCAAGTATTTAATTACTATGACIGCTCCCCAGCCCTGGCTCTGCAATGGGCACCTGGGATGAGCCCGCTGTGAGCCCTCG 910  
 890 930

Fig. 2-3

950 TCC TGAGG GTCC CCA CCGG ACCCTT GAGAGTATCA GGTCTCC CACGTGG GAGACA AAGAAATCC CTGTTTAATATTTA 990 1010  
 1030 AACAGCAGTGTTC CCCCATCTGGGTCCTTG CACCCTCACTCTGG CCTCAGCCGACTGCACAGCGGCC CCTGCATCC CCTT 1070 1090  
 1110 GGCCTGTGAGG CCCCCTGGACAAGCAGAGGTGGCCAGAGCTGGGAGGGCATG GCCCTGGGGTCCCACGAATTTGCTGGGGA 1150 1170  
 1190 TCTCGTTTTCTCTTAAGACTTTTGGGACATGTTTGACTCCCGAACATCACCGACCGGTCGCCGTCTCCGTGTTTCTGGGTG 1230 1250  
 1270 GCTCGGACACCTGCCCTGCCCCALGAGGGTCAGGACTGTGACTCTTTAGGGCCAGGAGGTGCCTGGACATTTG 1310 1330  
 1350 CCTTGCTGGACGGGGACTGGGGATCTGGGAGGGAGCAGACAGGAGGAATCATGTCAGGCCCTGTGTGTGAAGGAAGCTC 1370 1410  
 1430 CACTGTCACCCCTCCACTCTTLCACCCCCCACTCACCAGTGTCCCCTCCACTGTACATTGTAACTGAACCTCAGGATA 1450 1490  
 1510 ATAAAGTGTTCCTCCAAAAA AAAAAAAAAAAAAAAAAAAAA 1530

Fig. 3-1

GGAGCCTGCAGCCCGCCAGCCCCACCCAGACCCC

|   |     |     |     |
|---|-----|-----|-----|
| ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG AAG CTG ATG ACC CTG CAG CTG CTG CTG TGG | 30  | 70  | 90  |
| Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys Leu Met Ala Leu Gln Leu Leu Leu Trp | -30 | -20 |     |
| CAC AGT GCA CTC TGG ACA GTG CAG GAA GCC ACC CCC CTG GGC CCT GCC AGC TCC CTG CCC | 110 | 130 | 150 |
| His Ser Ala Leu Trp Thr Val Gln Glu Ala Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro | -10 | -1  | 10  |
| CAG AGC TTC CTG CTC AAG TGC TTA GAG CAA GTG AGG AAG ATC CAG GGC GAT GGC GCA GCG | 170 | 190 | 210 |
| Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala |     | 20  | 30  |
| CTC CAG GAG AAG CTG TGT GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG GTG CTG CTC | 230 | 250 | 270 |
| Leu Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu |     | 40  | 50  |
| GGA CAC TCT CTG GGC ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC AGC CAG GCC CTG CAG | 290 | 310 | 330 |
| Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln |     | 60  | 70  |
| CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC GGC CTT TTC CTC TAC CAG GGG CTC CTG CAG | 350 | 370 | 390 |
| Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln |     | 80  | 90  |

Fig. 3-2

410  
 GCC CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC ACC TTG GAC ACA CTG CAG CTG GAC GTC 450  
 Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val 110  
 100  
 430  
 470  
 GCC GAC TTT GCC ACC ACC ATC Thr Ile Trp Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu 510  
 Ala Asp Phe Ala Thr Thr Ile Trp Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu 130  
 120  
 530  
 CAG CCC ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC CGG GCA GGA GGG 570  
 Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly 150  
 140  
 590  
 GTC CTA GTT GCC TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG TAC CGC GTT CTA CGC CAC 630  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His 170  
 160  
 650  
 CTT GCC CAG CCC TGA GCCAAGCCCTCCCCATCCCATGATTAICTCTAATTTAATATTTAIGTCIATTTAAGCC 690  
 Leu Ala Gln Pro End  
 710  
 730  
 TCATATTTAAAGACAGGGGAGCAGAACCGAGGCCCCAGCCCTCTGTGTCCCTCCCTGCCAATTTCTGAGTTTCATCTCC 770  
 750  
 790  
 TGCCCTGTAGCAGTGAGAAAAGCCTCTGCTCCATCCCTGGACTGGGAGGTAGATAGGTAATACCAAGTATTAT 850  
 810  
 830



Fig. 3-3

<sup>870</sup>TACTATGACTCTCCCAGCCCTGGCTCTGCAATGGCACTGGATGAGCCGCTGTGAGCCCTGGTCCCTGAGGGTCCC  
<sup>910</sup>  
<sup>950</sup>CACCTGGGACCCCTGAGAGTATCAGGTCTCCCAGTGGGAGACAAAGAAATCCCTGTTTAAATATTTAAACAGCAGTGTTC  
<sup>1030</sup>  
<sup>1050</sup>CCCATCTGGGTCTTGACCCCTCAGCCGACTGCACAGGGCCCTGCATCCCTTGGCTGTGAGGCC  
<sup>1110</sup>  
<sup>1130</sup>CCTGGACAAGCAGAGGTGGCCAGAGCTGGGAGGCATGGCCCTGGGGTCCACAGAAATTTGCTGGGAAATCTCGTTTCT  
<sup>1190</sup>  
<sup>1210</sup>TCTTAAGACTTTGGGACATGGTTTGACTCCCGAATCACCAGCGCTCTCCCTGTTTCTGGGTGGCCTCGGGACA  
<sup>1270</sup>  
<sup>1290</sup>CCTGCCCTGCCCCACGAGGGTCAGGACTGTGACTCTTTTAGGGCCAGGCAGGTGCCCTGGACATTTGCCCTGTGCTGGAC  
<sup>1350</sup>  
<sup>1370</sup>GGGGACTGGGGATGTGGGAGGGAGCAGACAGGAAATCATGTGAGGCTGTGTGAAAGGAAAGCTCCACCTGTCACCC  
<sup>1430</sup>  
<sup>1450</sup>TCCACCTCTCACCCTCCACTCACCAGTGTCCCTCCACTGTCACATTTGTAAGTGAACCTCAGGATAATAAAGTGTCTG  
<sup>1490</sup>  
<sup>1510</sup>CCTCCAAAAAATAAAAAAAAAAAAAA

Fig. 4

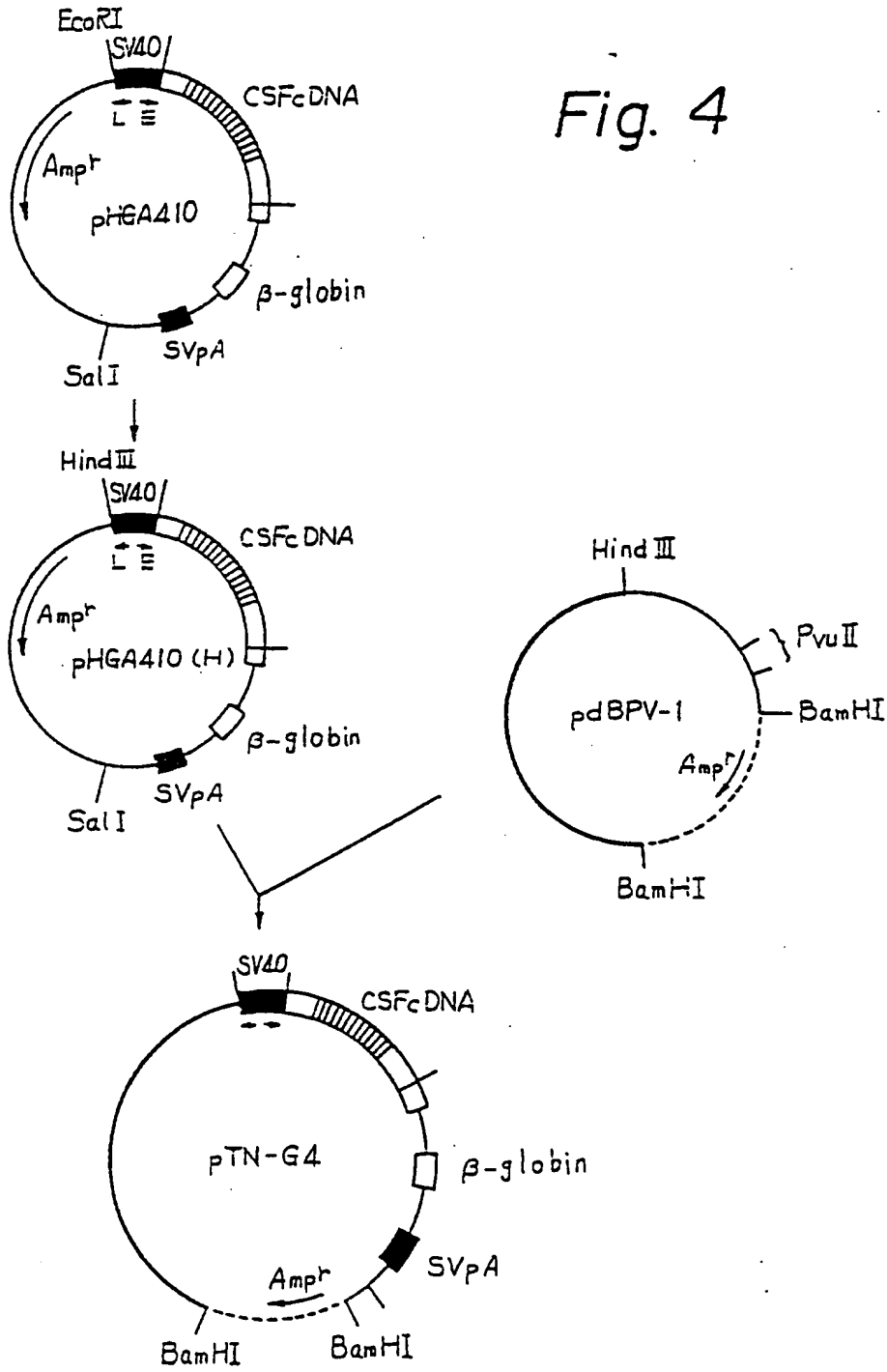


Fig. 5

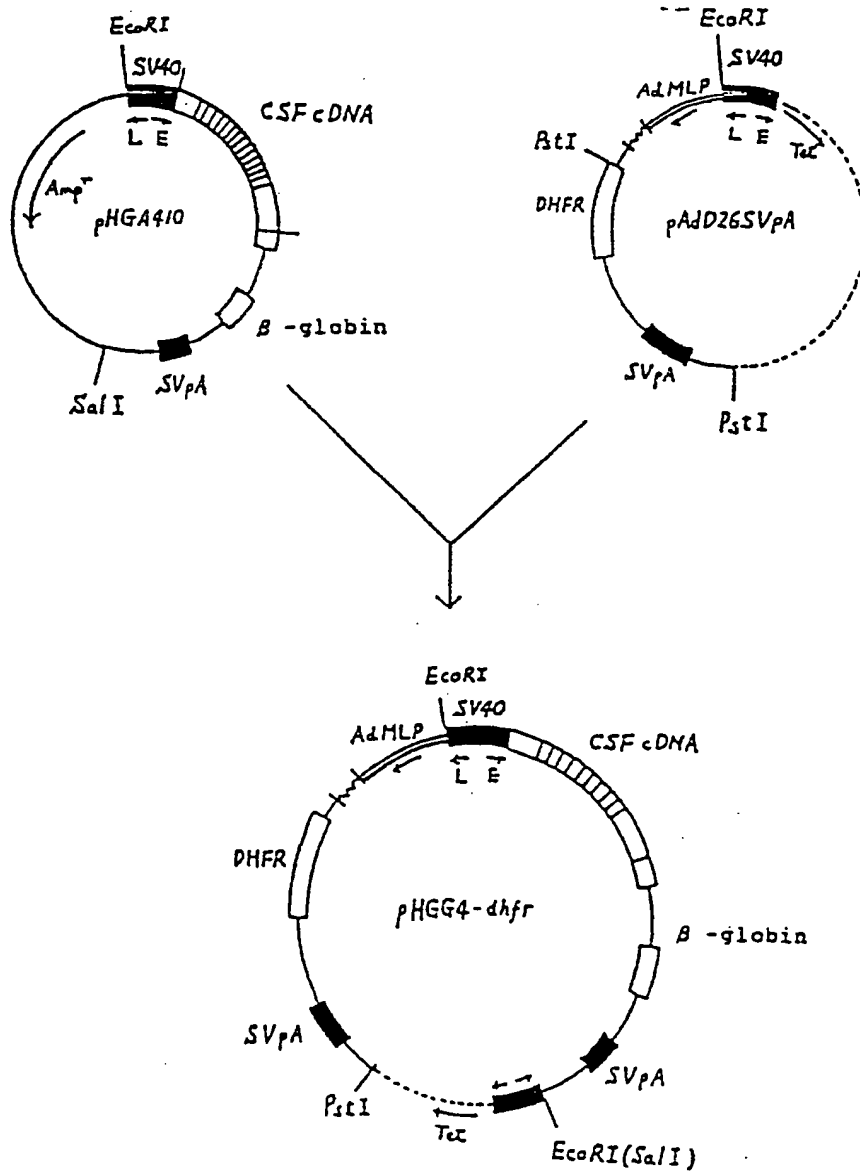


Fig. 6

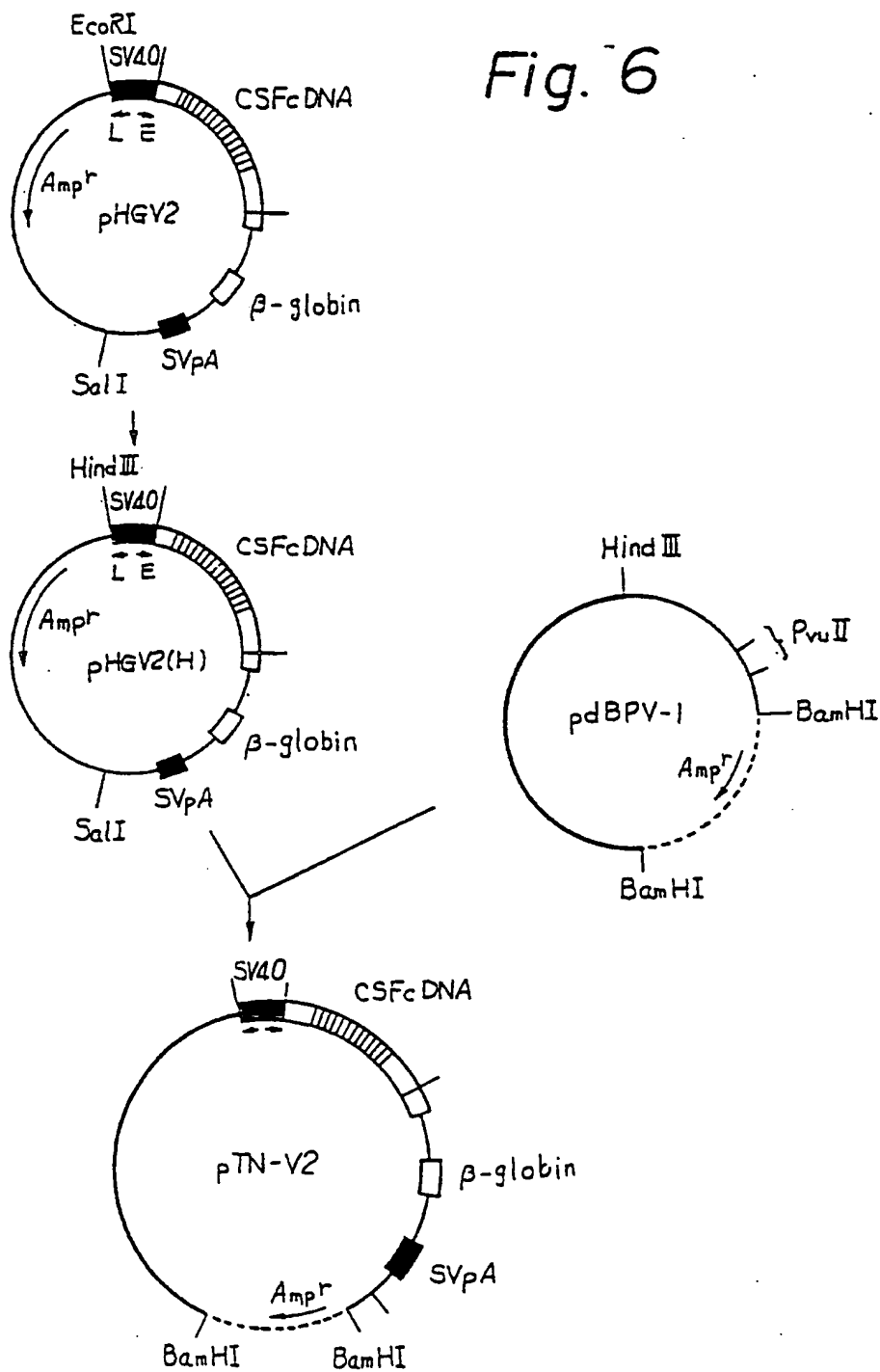
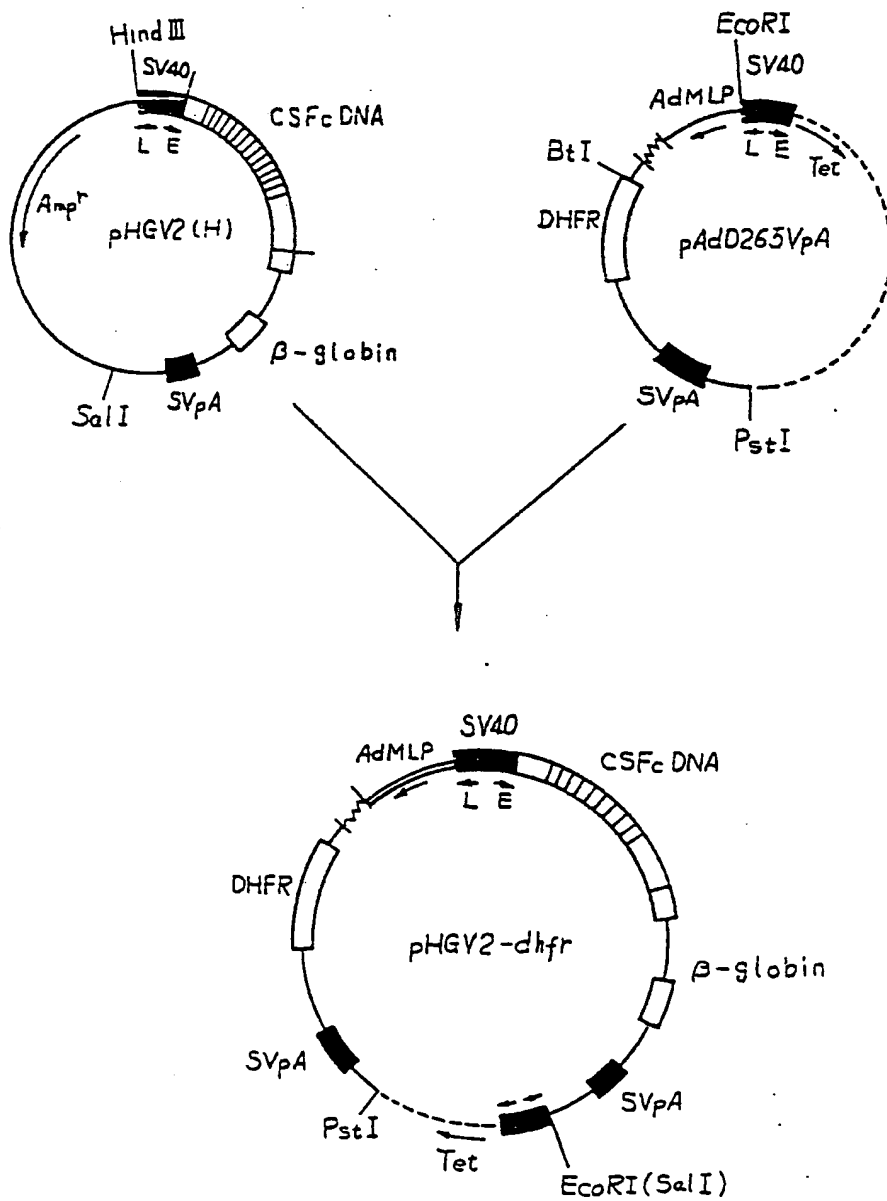


Fig. 7



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